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Use of osteoprotegerin for the prevention and/or treatment of fibrotic disease

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USE OF OSTEOPROTEGERIN FOR THE TREATMENT AND/OR
PREVENTION OF FIBROTIC DISEASE

FIELD OF THE INVENTION

5 The present invention is in the field of fibrotic diseases and connective tissue disorders. More specifically, the invention relates to the use of osteoprotegerin for the treatment and/or prevention of fibrotic diseases, in particular scleroderma.

BACKGROUND OF THE INVENTION

10 Scleroderma is a disease of the connective tissue characterized by fibrosis of the skin and internal organs, leading to organ failure and death (Black et al., 1998; Clements and Furst, 1996). Scleroderma has a spectrum of manifestations and a variety of therapeutic implications. It comprises localized scleroderma, systemic sclerosis, scleroderma-like disorders, and Sine scleroderma (Smith, 2000). Whilst
15 localized scleroderma is a rare dermatologic disease associated with fibrosis and manifestations limited to skin, systemic sclerosis is a multisystem disease with variable risk for internal organ involvement and variation in the extent of skin disease. Systemic sclerosis can be diffuse or limited. Limited systemic sclerosis is also called
20 CREST (calcinosis, Raynaud's esophageal dysfunction, sclerodactyly, telangiectasiae). Scleroderma-like disorders are believed to be related to industrial environment exposure. In Sine disease, there is internal organ involvement without skin changes.

 The major manifestations of scleroderma and in particular of systemic sclerosis are inappropriate excessive collagen synthesis and deposition, endothelial
25 dysfunction, spasm, collapse and obliteration by fibrosis.

 Scleroderma is a rare disease with a stable incidence of approximately 19 cases per 1 million persons. The cause of scleroderma is unknown. However, the genetic predisposition is important. Abnormalities involve autoimmunity and alteration of endothelial cell and fibroblast function. Indeed, systemic sclerosis is probably the
30 most severe of the auto-immune diseases with a reported 50% mortality within 5 years of diagnosis (Silman, 1991).

 In terms of diagnosis, an important clinical parameter is skin thickening proximal to the metacarpophalangeal joints. Raynaud's phenomenon is a frequent, almost universal component of scleroderma. It is diagnosed by color changes of the
35 skin upon cold exposure. Ischemia and skin thickening are symptoms of Raynaud's disease.

Several underlying biological processes are implicated in the initiation, severity and progression of the disease and include vascular dysfunction, endothelial cell activation and damage, leukocyte accumulation, auto-antibody production and crucially, an uncontrolled fibrotic response which may lead to death (Clements and Furst, 1996). Fibroblasts have a pivotal role in the pathogenesis of this disease. Primary fibroblasts obtained from patients with scleroderma exhibit many of the characteristic properties of the disease seen in vivo, notably increased extracellular matrix synthesis and deposition, notably of collagen and fibronectin, and altered growth factor and cytokine production such as of TGF β and CTGF (Strehlow and Korn, 1998 and LeRoy, 1974).

There is no curative treatment of scleroderma. Innovative but high-risk therapy proposed autologous stem cell transplantation (Martini et al., 1999). In particular, there are currently no treatments for scleroderma targeting the fibrotic process (Wigley and Boling, 2000).

Identification of the genes associated with disease risk and scleroderma progression may lead to the development of effective strategies for intervention at various stages of the disease.

Osteoprotegerin (OPG) was first identified in 1997 as a novel cytokine secreted by fibroblasts (Simonet et al., 1997). Human OPG is a 401 amino acid protein and containing a signal peptide of 21 amino acids, that is cleaved before glutamic acid 22 giving rise to a 380 amino acid mature protein. Thus, OPG is a soluble protein. It is a member of the TNF receptor family (Morinaga et al., 1998, Yasuda et al., 1998), comprising four cysteine-rich TNFR like domains in its N-terminal portion (Simonet et al., 1997). OPG has been shown to have a role in the development of bone, and mice lacking the OPG gene had an osteoporotic phenotype and gross skeletal abnormalities (Min et al., 2000).

Osteoprotegerin, which is produced by osteoblasts and bone marrow stromal cells, lacks a transmembrane domain and acts as a secreted decoy receptor which has no direct signaling capacity. OPG acts by binding to its natural ligand osteoprotegerin ligand (OPGL), which is also known as RANKL (receptor activator of NF-kappaB ligand). The binding between OPG and OPGL binding prevents OPGL from activating its cognate receptor RANK, which is the osteoclast receptor vital for osteoclast differentiation, activation and survival.

Human OPG is member of the TNFR family and is a single-copy gene that consists of 5 exons and spans 29 kb of the genome (Simonet et al., 1997). Recombinant OPG exists in monomeric and dimeric forms of apparent molecular

weights of 55 and 110 kDa, respectively (Simonet et al., 1997). Truncation of the N-terminal domain to cysteine 185 produces inactivation, presumably by disruption of the SS3 disulfide bond of the TNFR-like domain, whereas truncation of the C-terminal portion of the protein to amino acid 194 did not alter biological activity. Thus, the N-terminal TNFR-like domain of OPG is sufficient to prevent osteoclastogenesis (Simonet et al., 1997).

Overexpression of OPG in transgenic mice leads to profound osteopetrosis secondary to a near total lack of osteoclasts. Conversely, ablation of the OPG gene causes severe osteoporosis in mice. Ablation of OPGL or RANK also produces profound osteopetrosis, indicating the important physiological role of these proteins in regulating bone resorption. The secretion of OPG and OPGL from osteoblasts and stromal cells is regulated by numerous hormones and cytokines, often in a reciprocal manner. The relative levels of OPG and OPGL production are thought to ultimately dictate the extent of bone resorption. Excess OPGL increases bone resorption, whereas excess OPG inhibits resorption. Recombinant OPG blocks the effects of virtually all factors, which stimulate osteoclasts, in vitro and in vivo. OPG also inhibits bone resorption in a variety of animal disease models, including ovariectomy-induced osteoporosis, humoral hypercalcemia of malignancy, and experimental bone metastasis. Therefore, OPG might represent an effective therapeutic option for diseases associated with excessive osteoclast activity (Kostenuik and Shalhoub, 2001).

However, osteoprotegerin has not yet been suggested to be involved in fibrotic diseases.

25

SUMMARY OF THE INVENTION

The invention is based on the finding that administration of osteoprotegerin results in a significant amelioration of the disease in an established animal model of lung fibrosis. Lung fibrosis is one of the manifestations of scleroderma.

It is therefore a first object of the invention to use osteoprotegerin for the preparation of a medicament for the treatment and/or prevention of fibrotic diseases, in particular of scleroderma. It is a second object of the invention to use a cell expressing osteoprotegerin, or an expression vector comprising the coding sequence of osteoprotegerin, for the preparation of a medicament for the treatment and/or prevention of a fibrotic disease, in particular systemic sclerosis. Pharmaceutical compositions comprising osteoprotegerin and further anti-fibrotic drugs, such as halofuginone, and methods of treatment comprising administering osteoprotegerin to the human body are also within the scope of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

- Fig. 1 Expression of OPG mRNA in normal (hatched) and diseased (hatched horizontally) fibroblasts from 6 scleroderma patients determined by gene filter microarray analysis. The mean expression level is given in (a) and the median is given in (b).
- Fig. 2 Real time PCR analysis of OPG mRNA in 9 scleroderma patients. For each patient, results are expressed as the ratio of expression of OPG mRNA in lesional/non-lesional fibroblasts.
- Fig. 3 OPG mRNA expression in biopsies taken from lesional and non-lesional skin in 5 scleroderma patients determined by real time PCR. Bars denote the median expression value for each group.
- Fig. 4 Western blot analysis of OPG expression in lesional and normal skin fibroblasts. Lesional fibroblasts (A1-A4) and normal fibroblasts (N1-N4). Arrows on the left hand side of the gel indicate the position of native OPG (a 55 kDa monomer) and its dimeric form (110 kDa). OPG Fc fusion protein (purchased from R&D systems) was used as a positive control for the antibody.
- Fig. 5 Effect of OPG on collagen synthesis in AG1518 human fibroblasts. AG1518 cells were either completely untreated or pretreated with 10, 20 or 40 ng/ml OPG for 24 h, or with 40 ng OPG + anti OPG neutralising monoclonal antibody (1 μ g/ml), or with anti OPG neutralising monoclonal antibody alone followed by 2 ng/ml TGF β 1 for 24 h. Collagen synthesis was determined by ELISA. Results are the mean of triplicate determinations + S.E.M.
- Fig. 6 Effect of OPG transfection on type I collagen α 2 synthesis in human primary fibroblasts (OBHC cells). OBHC cells were transfected with pcDNA3.1/OPG (OPG) or pcDNA3.1 alone (mock) as described in methods. Collagen in conditioned medium was measured 24 h after TGF β 1 treatment by ELISA. Results are the mean of triplicate determinations \pm S.E.M.
- Fig. 7 Effect of OPG on type 1 collagen α 2 promoter activity in mouse embryo fibroblasts. Mouse embryo fibroblasts (π S3 cells) were transfected with pGL3 vector containing 3.5 kb of collagen 1 α 2 promoter linked to luciferase cDNA.

After transfection, cells were transferred to fresh medium and were either untreated (control) or treated with of halofuginone (10^{-10} M) alone (HF); TGF β 1 (5 ng/ml) (TGF β 1); or TGF β 1 (5 ng/ml) + HF (10^{-8} M) (HF+TGF β 1) for 12 h with increasing concentrations of osteoprotegerin. Results are the mean of triplicate determinations. * denotes $P < 0.05$ and ** denotes $P < 0.01$.

Fig. 8 Reverse transcriptase PCR analysis of osteoprotegerin mRNA after *in vitro* treatment of lesional and non-lesional fibroblasts from scleroderma patients with halofuginone. One % agarose – ethidium bromide stained gel showing RT PCR reaction products for osteoprotegrin (top) and GAPDH (bottom) in lesional (suffix A), or non-lesional (suffix N), fibroblasts.

Fig. 9 Effect of *in vivo* administration of osteoprotegerin on the development of lung fibrosis in bleomycin treated mice. A: Change in mouse body mass following treatment with osteoprotegerin. Results are expressed as the mean mass in g/group measured each day after intra-tracheal instillation of bleomycin. B: Mortality resulting from bleomycin administration. Results are expressed as a percentage of the number of deaths per group of 10 animals.

Fig. 10 Effect of *in vivo* administration of osteoprotegerin on the development of lung fibrosis in bleomycin treated mice. A: Extent of fibrosis in the lungs measured 12 days after bleomycin administration. Lungs were stained with trichrome. Results are expressed as the % of a representative lung lobe affected by fibrosis for each surviving animal. B: Extent of collagen deposition in the lungs measured 12 days after bleomycin administration. Hydroxyproline content was measured in a representative lung lobe taken from each surviving animal.

DESCRIPTION OF THE INVENTION

In accordance with the present invention, DNA gene filter microarray technology was used to identify differentially expressed genes in skin fibroblasts from fibrotic lesions obtained from patients with systemic sclerosis (scleroderma) compared to normal skin fibroblasts from the same patients. One gene, which was consistently down regulated in the scleroderma fibroblasts was osteoprotegerin (OPG), also called osteoclastogenesis inhibitory factor. Abnormal fibroblasts expressed significantly lower levels OPG mRNA compared to normal fibroblasts in 7 out of 9 patients tested. These results were corroborated by real time PCR analysis. In addition, real time RT-PCR analysis of total RNA isolated from whole biopsy specimens of abnormal skin from scleroderma patients indicated lower levels of OPG mRNA compared to clinically normal, age, sex and anatomical site matched control

biopsies. OPG protein was also decreased in lesional fibroblasts compared to normal fibroblasts isolated from the same patients. *In vitro*, OPG is able to decrease TGF β mediated collagen synthesis following transfection of fibroblasts with OPG cDNA or following treatment with the recombinant protein.

5 These *in vitro* data were further supported by *in vivo* data in an established animal model. Administration of osteoprotegerin reduced lung fibrosis and decreased collagen deposition in the bleomycin-induced lung fibrosis model. At the histological level, the lungs of osteoprotegerin treated animals resembled those of naïve, untreated mice.

10 Therefore, the invention relates to the use of a substance for the manufacture of a medicament for the treatment and/or prevention of a fibrotic disease, wherein the substance is selected from the group consisting of:

- a) A polypeptide comprising SEQ ID NO: 2 or SEQ ID NO: 4;
- b) A polypeptide comprising amino acids 22 to 401 of SEQ ID NO: 2 or SEQ ID NO: 4;
- 15 c) A polypeptide comprising one, two, three or four cysteine-rich domains of osteoprotegerin;
- d) A polypeptide comprising amino acids 22 to 194 of SEQ ID NO: 2 or SEQ ID NO: 4;
- 20 e) A mutein of any of (a) to (d), wherein the amino acid sequence has at least 40 % or 50 % or 60 % or 70 % or 80 % or 90 % identity to at least one of the sequences in (a) to (d);
- f) A mutein of any of (a) to (d) which is encoded by a DNA sequence which hybridizes to the complement of the DNA sequence encoding any of (a) to (d) under moderately
- 25 stringent conditions or under high stringent conditions;
- g) A mutein of any of (a) to (d) wherein any changes in the amino acid sequence are conservative amino acid substitutions to the amino acid sequences in (a) to (d);
- h) a salt or an isoform, fused protein, functional derivative, active fraction or circularly permuted derivative of any of (a) to (g).

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It will be appreciated by the person skilled in the art that in accordance with the present invention, a substance which stimulates release or potentiates the activity of endogenous osteoprotegerin can equally be used for treatment and/or prevention of fibrotic disease, in particular of scleroderma. Said substance may be mature

35 osteoprotegerin itself, or any fragment of osteoprotegerin binding to OPGL and thereby preventing OPGL binding to RANK, and preventing initiating signaling through RANK. Such a substance may be e.g. an antibody directed to OPGL. It is

known that OPG binds to OPGL, and that this interaction prevents binding of OPGL to its receptor RANK. Therefore, the person skilled in the art will appreciate that any substance preventing the binding of OPGL to its receptor RANK, or any other agent blocking RANK activity or signalling, will have the same activity as osteoprotegerin in prevention and/or treatment of fibrotic disease, in particular of scleroderma. Agents blocking OPGL binding to RANK may be antagonistic antibodies directed to OPGL, for instance. Agents blocking RANK activity may further be antagonistic antibodies directed to RANK, for instance. Further agents blocking OPGL/RANK interaction may be chemical compounds interfering with binding of these two proteins to each other, for example, but also any other chemical or biological inhibitor of signalling through the RANK receptor.

The full length cDNA of human osteoprotegerin has been cloned and is depicted as SEQ ID NO: 1 of the attached sequence listing. The corresponding amino acid sequence is given as SEQ ID NO: 2 of the attached sequence listing. The sequences are described at www.ncbi.nlm.nih.gov under AB_008821, as well as by Morinaga et al. (1998). An isoform or polymorphic form of osteoprotegerin has been described by Simonet et al. (1997). The cDNA according to Simonet et al. (1997) is depicted as SEQ ID NO: 3 of the attached sequence listing, and the corresponding amino acid sequence is given as SEQ ID NO: 4. Both sequences are further available from www.ncbi.nlm.nih.gov under U94332. The only difference between the proteins found by Morinaga et al. and Simonet et al. concerns amino acid position 263, which may be an aspartic acid or an alanine, respectively.

The term "osteoprotegerin", as used herein, relates to any of the substances considered above in (a) to (h).

The term "treatment and/or prevention" as used herein encompasses any attenuation, reduction, or partial, substantial or complete prevention or blockage of disease formation, development, progression or of the formation, development or progression of any one or several or all of the symptoms of the disease.

The term "fibrotic disease" as used herein relates to diseases involving fibrosis, which may e.g. be due to chronic inflammation or repair and reorganization of tissues. Fibrosis may involve any organ of the human body, such as e.g. the skin, lung, pancreas, liver or kidney. Therefore, the invention also relates to treatment and/or prevention of fibrotic diseases such as liver cirrhosis, interstitial pulmonary fibrosis, Dupuytren's contracture, keloid and other scarring/wound healing abnormalities, postoperative adhesions and reactive fibrosis, as well as chronic heart failure, in particular after myocardial infarction. Further diseases or disorders

5 treatable with osteoprotegerin comprise wound healing diseases, in particular wound healing in the lung, comprising chronic inflammation of the lung and ultimately fibrosis or scarring of lung surfaces. Disorders involving inflammation of the lung comprise e.g. idiopathic pulmonary fibrosis, sarcoidosis, bronchopulmonary dysplasia, fibroproliferative ARDS, as well as pulmonary manifestations or systemic diseases such as rheumatoid arthritis (Krein et al., 2001).

10 Fibrosis generally involves generation or proliferation of connective tissue, which replaces functional specialized tissue of a given organ. Therefore, in a preferred embodiment of the present invention, the fibrotic disease is a connective tissue disease.

In a further preferred embodiment, the fibrotic disease is scleroderma.

15 The term "scleroderma" as used herein relates to a disease also called scleroderma, systemic sclerosis or systemic scleroderma. These terms are used synonymously within this patent application. Systemic sclerosis is a chronic disease of unknown cause, characterized by diffuse fibrosis; degenerative changes; and vascular abnormalities in the skin, articular structures, and internal organs (especially the esophagus, gastrointestinal tract, lung, heart, and kidney, for example). It may be localized, or mixed, systemic, limited or diffuse.

20 The term "scleroderma" preferably relates to localized, systemic, limited and diffuse scleroderma as well as overlap syndromes.

25 Localized scleroderma primarily affects the skin, but may also affect the underlying muscles and bones. However, it generally does not affect internal organs. Localized scleroderma is relatively mild, and may be related to systemic scleroderma in terms of similar superficial symptoms, such as the appearance of skin biopsy under the microscope.

30 Systemic scleroderma comprises several types of symptoms or groups of symptoms, such as CREST, limited and diffuse. It may also be referred to as progressive systemic sclerosis, or familial progressive systemic sclerosis. Systemic scleroderma may e.g. affect the skin, blood vessels, and/or internal organs. When it affects the skin, it can cause the skin to harden, most commonly on the hands and/or face. When it affects the blood vessels, it can cause Raynaud's disease. The most serious forms of systemic sclerosis affect the internal organs, and may cause disability or even death. Among others, systemic sclerosis comprises: scleroderma lung disease, scleroderma renal crisis, cardiac manifestations, muscular weakness including fatigue or limited CREST, gastrointestinal dysmotility and spasm, and abnormalities in the central, peripheral and autonomic nervous system. With regard

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to the nervous system abnormalities, carpal tunnel syndrome followed by trigeminal neuralgia are the most common.

Limited Scleroderma may e.g. be limited to the hands, although the face and neck may also be involved.

5 Diffuse Scleroderma comprises skin tightening and also occurs above the wrists (or elbows). There are several subcategories of diffuse systemic sclerosis, such as "scleroderma sine scleroderma" where there is internal organ fibrosis, but no skin tightening; and familial progressive systemic sclerosis, a rare form occurring in families.

10 Overlap syndromes are referred to if a scleroderma patient also has other autoimmune disease (such as lupus, rheumatoid arthritis, etc.), as e.g. in diffuse scleroderma in overlap with lupus. Scleroderma symptoms can also be a part of mixed connective tissue disease (MCTD), or Undifferentiated Connective Tissue Disease (UCTD).

15 The term "osteoprotegerin" as used herein, relates to a protein comprising all, or a portion of the sequence of SEQ ID NO: 2 or 4 (both human) of the enclosed sequence listing, as well as to salts, isoforms, muteins, active fractions, functional derivatives and circularly permuted derivatives thereof. OPG from species other than human, such as mouse or rat, may be used in accordance with the present
20 invention, as long as there is a sufficient identity between the proteins as to allow the protein to exhibit its biological activity, and without eliciting a substantial immune response in a human being.

 Preferably, the term "osteoprotegerin" refers to a mature protein lacking the signal peptide. The signal peptide comprises the N-terminal 21 amino acids of OPG
25 as defined in SEQ ID NO: 2 or 4, meaning that the mature protein comprises amino acids 22 to 401 of SEQ ID NO: 2 or 4. The term "osteoprotegerin", as used herein, further relates to any fragment, portion, domain or sub-domain of SEQ ID NO: 2 or 4 showing the desired activity in scleroderma or other fibrotic diseases. Protein
30 fragments or one or more domains of the protein may be used according to the invention, as long as they exhibit any beneficial effect on fibrotic disease, preferably an effect which is at least comparable of the full length protein. The beneficial effect can be measured in one of the *in vitro* or *in vivo* tests described in the examples below, or in any other assay adequate to demonstrate an effect in fibrotic diseases, in particular of scleroderma. For example, osteoprotegerin comprises a cysteine-rich
35 TNFR-like domain in its N-terminal portion, as described by Simonet et al., 1997. A fragment comprising this domain was shown to retain the biological activity of OPG in an *in vitro* test. Therefore, a preferred OPG fragment comprises the TNFR-like

domain of OPG, in particular a fragment comprising amino acids 22 to 194 of SEQ ID NO: 2 or 4.

In accordance with the present invention, osteoprotegerin can be a naturally occurring, i.e. native protein, or a recombinant protein. Recombinant production may be carried out in eukaryotic cells, such as yeast cells or mammalian cells, preferably
5 in CHO cells, HEK cells (human embryonic kidney cells) or in human fibroblast cells or cell lines. It may further be produced in prokaryotic cells such as *E. coli*.

Osteoprotegerin has been described to occur in monomeric and dimeric form (Simonet et al., 1997). Therefore, in accordance with the present invention, OPG
10 may be a monomer, a dimer or a multimer.

Preferably, osteoprotegerin is glycosylated at one or more sites. It may also be unglycosylated, depending on the given needs and the source of production or isolation of the protein.

The term "salts" herein refers to both salts of carboxyl groups and to acid
15 addition salts of amino groups of osteoprotegerin molecule or analogs thereof. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition
20 salts include, for example, salts with mineral acids, such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids, such as, for example, acetic acid or oxalic acid. Of course, any such salts must retain the biological activity of osteoprotegerin relevant to the present invention, i.e., exert a beneficial effect on fibrotic diseases, in particular scleroderma.

Isoforms or splice variants of osteoprotegerin may also be used according to
25 the invention, as long as they are capable of inhibiting disease progression and/or symptoms of that disease.

As used herein the term "muteins" refers to analogs of osteoprotegerin, in which one or more of the amino acid residues of natural osteoprotegerin are replaced
30 by different amino acid residues, or are deleted, or one or more amino acid residues are added to the natural sequence of osteoprotegerin, having preferably at least the same activity as wild type osteoprotegerin or even having a much more potent activity. The biological activity of OPG can e.g. be measured by assaying the binding of OPG to its natural ligand OPGL. Assays for assessing protein-protein interactions
35 are well known by the person skilled in the art. Examples for such assays are ELISA type binding assays, immuno-precipitation assays, or measurement in any other suitable system such as the BIAcore system. These muteins are prepared by known

synthesis and/or by site-directed mutagenesis techniques, or any other known technique suitable therefor.

Any such mutein preferably has a sequence of amino acids sufficiently duplicative of that of mature osteoprotegerin, such as to have at least a substantially similar activity of osteoprotegerin. The activity of a osteoprotegerin mutant can further
5 be tested in the assays explained in the examples below. Measuring the amount of collagen synthesis in fibroblasts treated with OPG may a suitable test for assessing the activity of osteoprotegerin muteins, for example.

Muteins in accordance with the present invention include proteins encoded by
10 a nucleic acid, such as DNA or RNA, which hybridizes to DNA or RNA, which encodes osteoprotegerin, in accordance with the present invention, under stringent conditions. The term "stringent conditions" refers to hybridization and subsequent washing conditions, which those of ordinary skill in the art conventionally refer to as "stringent". See Ausubel et al., Current Protocols in Molecular Biology, supra,
15 Interscience, N.Y., §§6.3 and 6.4 (1987, 1992), and Sambrook et al. (Sambrook, J. C., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Without limitation, examples of stringent conditions include washing conditions 12-20°C below the calculated T_m of the hybrid under study in, e.g., 2 x
20 SSC and 0.5% SDS for 5 minutes, 2 x SSC and 0.1% SDS for 15 minutes; 0.1 x SSC and 0.5% SDS at 37°C for 30-60 minutes and then, a 0.1 x SSC and 0.5% SDS at 68°C for 30-60 minutes. Those of ordinary skill in this art understand that stringency conditions also depend on the length of the DNA sequences, oligonucleotide probes (such as 10-40 bases) or mixed oligonucleotide probes. If mixed probes are used, it
25 is preferable to use tetramethyl ammonium chloride (TMAC) instead of SSC. See Ausubel, supra.

Any such mutein preferably has a sequence of amino acids sufficiently duplicative of that of osteoprotegerin, such as to have substantially similar, or even better, biological activity as osteoprotegerin.

One easily measurable activity of osteoprotegerin is its capability of reducing
30 collagen synthesis. As long as the mutein has substantial collagen reducing activity, it can be considered to have substantially similar activity to osteoprotegerin. Thus, it can be determined whether any given mutein has at least substantially the same activity as osteoprotegerin by means of routine experimentation comprising
35 subjecting such a mutein.

In a preferred embodiment, any such mutein has at least 40% identity or homology with the sequence of mature osteoprotegerin. More preferably, it has at

least 50%, at least 60%, at least 70%, at least 80% or, most preferably, at least 90% identity or homology thereto.

Identity reflects a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, determined by comparing the sequences. In general, identity refers to an exact nucleotide to nucleotide or amino acid to amino acid correspondence of the two polynucleotides or two polypeptide sequences, respectively, over the length of the sequences being compared.

For sequences where there is not an exact correspondence, a "% identity" may be determined. In general, the two sequences to be compared are aligned to give a maximum correlation between the sequences. This may include inserting "gaps" in either one or both sequences, to enhance the degree of alignment. A % identity may be determined over the whole length of each of the sequences being compared (so-called global alignment), that is particularly suitable for sequences of the same or very similar length, or over shorter, defined lengths (so-called local alignment), that is more suitable for sequences of unequal length.

Methods for comparing the identity and homology of two or more sequences are well known in the art. Thus for instance, programs available in the Wisconsin Sequence Analysis Package, version 9.1 (Devereux J et al, 1984), for example the programs BESTFIT and GAP, may be used to determine the % identity between two polynucleotides and the % identity and the % homology between two polypeptide sequences. BESTFIT uses the "local homology" algorithm of Smith and Waterman (1981) and finds the best single region of similarity between two sequences. Other programs for determining identity and/or similarity between sequences are also known in the art, for instance the BLAST family of programs (Altschul S F et al, 1990, Altschul S F et al, 1997, accessible through the home page of the NCBI at www.ncbi.nlm.nih.gov) and FASTA (Pearson W R, 1990; Pearson 1988).

Mutins of osteoprotegerin, which can be used in accordance with the present invention, or nucleic acids coding therefor, include a finite set of substantially corresponding sequences as substitution peptides or polynucleotides which can be routinely obtained by one of ordinary skill in the art, without undue experimentation, based on the teachings and guidance presented herein.

Preferred changes for mutins in accordance with the present invention are what are known as "conservative" substitutions. Conservative amino acid substitutions of osteoprotegerin polypeptides or proteins, may include synonymous amino acids within a group which have sufficiently similar physicochemical properties that substitution between members of the group will preserve the biological function of the molecule (Grantham, 1974). It is clear that insertions and deletions of amino

acids may also be made in the above-defined sequences without altering their function, particularly if the insertions or deletions only involve a few amino acids, e.g., under thirty, and preferably under ten, and do not remove or displace amino acids which are critical to a functional conformation, e.g., cysteine residues. Proteins and
 5 muteins produced by such deletions and/or insertions come within the purview of the present invention.

Preferably, the synonymous amino acid groups are those defined in Table I. More preferably, the synonymous amino acid groups are those defined in Table II; and most preferably the synonymous amino acid groups are those defined in Table
 10 III.

TABLE I

Preferred Groups of Synonymous Amino Acids

| | Amino Acid | Synonymous Group |
|----|------------|-----------------------------------|
| 15 | Ser | Ser, Thr, Gly, Asn |
| | Arg | Arg, Gln, Lys, Glu, His |
| | Leu | Ile, Phe, Tyr, Met, Val, Leu |
| | Pro | Gly, Ala, Thr, Pro |
| | Thr | Pro, Ser, Ala, Gly, His, Gln, Thr |
| 20 | Ala | Gly, Thr, Pro, Ala |
| | Val | Met, Tyr, Phe, Ile, Leu, Val |
| | Gly | Ala, Thr, Pro, Ser, Gly |
| | Ile | Met, Tyr, Phe, Val, Leu, Ile |
| | Phe | Trp, Met, Tyr, Ile, Val, Leu, Phe |
| 25 | Tyr | Trp, Met, Phe, Ile, Val, Leu, Tyr |
| | Cys | Ser, Thr, Cys |
| | His | Glu, Lys, Gln, Thr, Arg, His |
| | Gln | Glu, Lys, Asn, His, Thr, Arg, Gln |
| | Asn | Gln, Asp, Ser, Asn |
| 30 | Lys | Glu, Gln, His, Arg, Lys |
| | Asp | Glu, Asn, Asp |
| | Glu | Asp, Lys, Asn, Gln, His, Arg, Glu |
| | Met | Phe, Ile, Val, Leu, Met |
| 35 | Trp | Trp |

TABLE II

More Preferred Groups of Synonymous Amino Acids

| | Amino Acid | Synonymous Group |
|----|------------|-------------------------|
| | Ser | Ser |
| | Arg | His, Lys, Arg |
| | Leu | Leu, Ile, Phe, Met |
| 5 | Pro | Ala, Pro |
| | Thr | Thr |
| | Ala | Pro, Ala |
| | Val | Val, Met, Ile |
| | Gly | Gly |
| 10 | Ile | Ile, Met, Phe, Val, Leu |
| | Phe | Met, Tyr, Ile, Leu, Phe |
| | Tyr | Phe, Tyr |
| | Cys | Cys, Ser |
| | His | His, Gln, Arg |
| 15 | Gln | Glu, Gln, His |
| | Asn | Asp, Asn |
| | Lys | Lys, Arg |
| | Asp | Asp, Asn |
| | Glu | Glu, Gln |
| 20 | Met | Met, Phe, Ile, Val, Leu |
| | Trp | Trp |

TABLE III

Most Preferred Groups of Synonymous Amino Acids

| | Amino Acid | Synonymous Group |
|----|------------|------------------|
| 25 | Ser | Ser |
| | Arg | Arg |
| | Leu | Leu, Ile, Met |
| | Pro | Pro |
| 30 | Thr | Thr |
| | Ala | Ala |
| | Val | Val |
| | Gly | Gly |
| | Ile | Ile, Met, Leu |
| 35 | Phe | Phe |
| | Tyr | Tyr |
| | Cys | Cys, Ser |

| | |
|-----|---------------|
| His | His |
| Gln | Gln |
| Asn | Asn |
| Lys | Lys |
| Asp | Asp |
| Glu | Glu |
| Met | Met, Ile, Leu |
| Trp | Met |

10 Examples of production of amino acid substitutions in proteins which can be
 used for obtaining muteins of osteoprotegerin polypeptides or proteins, for use in the
 present invention include any known method steps, such as presented in US patents
 4,959,314, 4,588,585 and 4,737,462, to Mark et al; 5,116,943 to Kothe et al.,
 4,965,195 to Namen et al; 4,879,111 to Chong et al; and 5,017,691 to Lee et al; and
 15 lysine-substituted proteins presented in US patent No. 4,904,584 (Shaw et al).

 The term "fused protein" refers to a polypeptide comprising osteoprotegerin,
 or a mutein thereof, fused with another protein, which, e.g., has an extended
 residence time in body fluids. Fusion proteins comprising all or a functional part of
 osteoprotegerin fused to all or a functional part of a protein capable of improving the
 20 biological activities of the molecule, like half-life in the human body, for instance, are
 preferred according to the invention. In a preferred embodiment the fused protein
 comprises an immunoglobulin (Ig) fusion. Fusion proteins comprising all or part of
 osteoprotegerin fused to all or part of an immunoglobulin are highly preferred. They
 can be monomeric or multimeric, hetero- or homomultimeric. Advantageously, the
 25 fused protein comprises the constant region of an immunoglobulin, in particular of the
 Fc portion of the immunoglobulin. Embodiments in which the immunoglobulin is of
 the IgG1 or IgG2 isotype are further preferred according to the invention. Preferably,
 the fusion is an Fc fusion.

 Osteoprotegerin may thus be fused to another protein, polypeptide or the like,
 30 e.g., an immunoglobulin or a fragment thereof. The fusion may be direct, or via a
 short linker peptide which can be as short as 1 to 3 amino acid residues in length or
 longer, for example, 13 amino acid residues in length. Said linker may be a tripeptide
 of the sequence E-F-M (Glu-Phe-Met), for example, or a 13-amino acid linker
 sequence comprising Glu-Phe-Gly-Ala-Gly-Leu-Val-Leu-Gly-Gly-Gln-Phe-Met
 35 introduced between the osteoprotegerin sequence and the immunoglobulin
 sequence.

"Functional derivatives" as used herein cover derivatives of osteoprotegerin, and their muteins and fused proteins, which may be prepared from the functional groups which occur as side chains on the residues or the N- or C-terminal groups, by means known in the art, and are included in the invention as long as they remain pharmaceutically acceptable, i.e. they do not destroy the activity of the protein which is at least substantially similar to the activity of osteoprotegerin, and do not confer toxic properties on compositions containing it. Therefore, in a preferred embodiment the functional derivative comprises at least one moiety attached to one or more functional groups, which occur as one or more side chains on the amino acid residues.

In accordance with the present invention, polyethylene glycol (PEG) side-chains are highly preferred moieties. PEG side chains may mask antigenic sites and extend the residence of the substance they are attached to in body fluids. Other derivatives include aliphatic esters of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives of free amino groups of the amino acid residues formed with acyl moieties (e.g. alkanoyl or carbocyclic aroyl groups) or O-acyl derivatives of free hydroxyl groups (for example that of seryl or threonyl residues) formed with acyl moieties.

"Active fractions" of osteoprotegerin and its muteins and fused proteins, cover any fragment or precursors of the polypeptide chain of the protein molecule alone or together with associated molecules or residues linked thereto, e.g., sugar or phosphate residues, or aggregates of the protein molecule or the sugar residues by themselves, provided said active fraction has at least a substantially similar activity to osteoprotegerin.

The invention further relates to the use of a nucleic acid molecule for manufacture of a medicament for the treatment and/or prevention of scleroderma, wherein the nucleic acid molecule comprises a nucleic acid sequence encoding a polypeptide comprising an amino acid sequence selected from the group consisting of:

- a) A polypeptide comprising SEQ ID NO: 2 or SEQ ID NO: 4;
- b) A polypeptide comprising amino acids 22 to 401 of SEQ ID NO: 2 or SEQ ID NO: 4;
- c) A polypeptide comprising one, two, three or four cysteine-rich domains of osteoprotegerin;
- d) A polypeptide comprising amino acids 22 to 194 of SEQ ID NO: 2 or SEQ ID NO: 4;

e) A mutein of any of (a) to (d), wherein the amino acid sequence has at least 40 % or 50 % or 60 % or 70 % or 80 % or 90 % identity to at least one of the sequences in (a) to (d);

5 f) A mutein of any of (a) to (d) which is encoded by a DNA sequence which hybridizes to the complement of the native DNA sequence encoding any of (a) to (d) under moderately stringent conditions or under high stringent conditions;

g) A mutein of any of (a) to (d) wherein any changes in the amino acid sequence are conservative amino acid substitutions to the amino acid sequences in (a) to (d);

10 h) An isoform, fused protein or active fraction of any of (a) to (g).

for the manufacture of a medicament for the treatment and/or prevention of fibrotic diseases. The invention preferably relates to the use of said nucleic acid molecules for the treatment and/or prevention of connective tissue diseases, and in particular of scleroderma.

15 In accordance with the present invention, osteoprotegerin may also be administered to the human body in form of a vector comprising said nucleic acid molecule. Therefore, the invention further relates to the use of a vector comprising said nucleic acid molecule for the manufacture of a medicament for the treatment and/or prevention of scleroderma or another fibrotic disorder. Preferably, the vector is an expression vector, comprising a promoter operably linked to all or part of the coding sequence of osteoprotegerin. In a further preferred embodiment, the vector is a gene therapy vector. Gene therapy vectors are known in the art, most of them are virally derived vectors, such as adenoviral or lentiviral vectors.

25 According to the invention, osteoprotegerin may also be administered to the human body in form of a cell producing and/or secreting osteoprotegerin. Therefore, the invention further relates to the use of a cell expressing osteoprotegerin for the manufacture of a medicament for the treatment and/or prevention of scleroderma or any other fibrotic disease, i.e. to cell therapy for the treatment and/or prevention of scleroderma or other fibrotic diseases. The cell may be a naturally producing osteoprotegerin and/or a transfected cell that produces recombinant osteoprotegerin. Preferred are cells expressing and secreting high amounts of the protein, such as over-expressing cells carrying high copy numbers of an expression vector comprising a nucleic acid molecule encoding osteoprotegerin.

30 As fibroblasts represent the machinery of fibrosis they are the most suitable cells for anti-fibrotic and scleroderma therapy. Therefore, preferably, osteoprotegerin expressing fibroblasts are used in accordance with the present invention.

The invention further relates to a cell comprising a vector comprising a nucleic acid molecule encoding all or part of osteoprotegerin for the preparation of a

medicament for treatment and/or prevention of fibrotic disease, in particular of scleroderma. A cell that has been genetically modified to produce a polypeptide according to the invention is also within the scope of the present invention.

The use of an expression vector for inducing and/or enhancing the
 5 endogenous production of osteoprotegerin in a cell normally silent or expressing amounts of the inhibitor which are not sufficient, are also contemplated according to the invention. Thus, the invention makes use of a technology known as endogenous gene activation (EGA) for the production of the desired protein.

10 Several combined treatments are preferred in accordance with the present invention. Therefore, preferably, the medicament of the invention further comprises:

- Interferon, in particular interferon- β
- A Tumor Necrosis Factor (TNF) antagonist, in particular soluble TNFRs, such as soluble p55 (TBPI) and/or soluble p75 (TBP II);
- 15 • A further anti-scleroderma agent;
- An anti-scleroderma agent selected from the group consisting of Halofuginone, ACE inhibitors, calcium channel blockers, proton pump inhibitors, NSAIDs, COX-inhibitors, corticosteroids, tetracycline, pentoxifylline, bucillamine, geranylgeranyl transferase inhibitors, rotterlin, prolyl-4-hydroxylase inhibitors, c-proteinase inhibitors, lysyl-oxidase
 20 inhibitors, relaxin, halofuginone, prostaglandins, prostacyclins, endothelin-1, nitric oxide, angiotensin II inhibitors and anti-oxidants.

All treatments are intended for simultaneous, sequential or separate use.

Pharmaceutical compositions comprising one or more of the above
 25 substances, together with osteoprotegerin, are within the scope of the present invention.

Although there is presently no cure for scleroderma, several agents or treatments are presently being used to treat scleroderma symptoms. Such anti-scleroderma agents, which may be used as combination therapy according to the
 30 invention, are summarized e.g. in Leighton (2001) or Wigley and Sule (2001), which are fully incorporated by reference herein.

Interferons are predominantly known for inhibitory effects on viral replication and cellular proliferation. Interferon- γ , for example, plays an important role in promoting immune and inflammatory responses. Interferon β (IFN- β , an interferon
 35 type I), is said to play an anti-inflammatory role.

In yet a further embodiment of the invention, osteoprotegerin is used in combination with a TNF antagonist. TNF antagonists exert their activity in several ways. First, antagonists can bind to or sequester the TNF molecule itself with sufficient affinity and specificity to partially or substantially neutralise the TNF epitope or epitopes responsible for TNF receptor binding (hereinafter termed "sequestering antagonists"). A sequestering antagonist may be, for example, an antibody directed against TNF.

Alternatively, TNF antagonists can inhibit the TNF signalling pathway activated by the cell surface receptor after TNF binding (hereinafter termed "signalling antagonists"). TNF antagonists are easily identified and evaluated by routine screening of candidates for their effect on the activity of native TNF on susceptible cell lines in vitro, for example human B cells, in which TNF causes proliferation and immunoglobulin secretion. The assay contains TNF formulation at varying dilutions of candidate antagonist, e.g. from 0,1 to 100 times the molar amount of TNF used in the assay, and controls with no TNF or only antagonist (Tucci et al., 1992).

Sequestering antagonists are the preferred TNF antagonists to be used according to the present invention. Amongst sequestering antagonists, those polypeptides that bind TNF with high affinity and possess low immunogenicity are preferred. Soluble TNF receptor molecules and neutralising antibodies to TNF are particularly preferred. For example, soluble forms of TNF-RI (p55) and TNF-RII (p75) are useful in the present invention. Truncated forms of these receptors, comprising the extracellular domains of the receptors or functional portions thereof, are more particularly preferred antagonists according to the present invention. Truncated soluble TNF type-I and type-II receptors are described in EP914431, for example.

Truncated forms of the TNF receptors are soluble and have been detected in urine and serum as about 30 kDa or 40 kDa TNF inhibitory binding proteins, which are called TBPI and TBPII, respectively (Engelmann et al., 1990). The simultaneous, sequential, or separate use of osteoprotegerin with the TNF antagonist and /or an Interferon is preferred, according to the invention.

According to the invention, TBPI and TBPII are preferred TNF antagonists to be used in combination with an osteoprotegerin. Derivatives, fragments, regions and biologically active portions of the receptor molecules functionally resemble the receptor molecules that can also be used in the present invention. Such biologically active equivalent or derivative of the receptor molecule refers to the portion of the polypeptide, or of the sequence encoding the receptor molecule, that is of sufficient

size and able to bind TNF with such an affinity that the interaction with the membrane-bound TNF receptor is inhibited or blocked.

In a further preferred embodiment, human soluble TNF-RI (TBPI) is the TNF antagonist to be used according to the invention. The natural and recombinant
5 soluble TNF receptor molecules and methods of their production have been described in the European Patents EP 308 378, EP 398 327 and EP 433 900.

Whilst it may be beneficial to block TNF- α in early stages of the disease, it has been discussed that in later stages, TNF itself may exert a beneficial effect on scleroderma (Abraham et al., 2000). Therefore, the invention further relates to a
10 combination of osteoprotegerin and TNF for treatment or prevention of scleroderma, in particular in advanced stages of disease. TNF- α or TNF- β may be used in accordance with the invention.

The invention further relates to a pharmaceutical composition comprising osteoprotegerin, optionally together with one or more pharmaceutically acceptable
15 carriers, diluents or excipients, for the treatment and/or prevention of fibrotic disease, in particular scleroderma. The pharmaceutical composition may further comprise any of the above-identified further components, and in particular an interferon, a TBP or a COX inhibitor.

The pharmaceutical composition according to the invention may also
20 comprise a vector comprising a nucleic acid molecule according to the invention, or a cell expressing osteoprotegerin.

The active ingredients of the pharmaceutical, i.e. polypeptides, nucleic acids or cells according to the invention, or combinations thereof, as well as the combinations of substances mentioned above, may be administered to an individual
25 in a variety of ways. The routes of administration include intradermal, transdermal (e.g. in slow release formulations), intramuscular, intraperitoneal, intravenous, subcutaneous, oral, epidural, topical, and intranasal routes. Any other therapeutically efficacious route of administration can be used, for example absorption through epithelial or endothelial tissues or by gene therapy wherein a DNA molecule
30 encoding the active agent is administered to the patient (e.g. via a vector), which causes the active agent to be expressed and secreted in vivo. In addition, the protein(s) according to the invention can be administered together with other components of biologically active agents such as pharmaceutically acceptable surfactants, excipients, carriers, diluents and vehicles.

35 The definition of "pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with effectiveness of the biological activity of the

active ingredient and that is not toxic to the host to which it is administered. For example, for parenteral administration, the active protein(s) may be formulated in a unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution.

5 For parenteral (e.g. intravenous, subcutaneous, intramuscular) administration, the active protein(s) can be formulated as a solution, suspension, emulsion or lyophilised powder in association with a pharmaceutically acceptable parenteral vehicle (e.g. water, saline, dextrose solution) and additives that maintain isotonicity (e.g. mannitol) or chemical stability (e.g. preservatives and buffers). The formulation
10 is sterilized by commonly used techniques.

The bioavailability of the active protein(s) according to the invention can also be ameliorated by using conjugation procedures which increase the half-life of the molecule in the human body, for example linking the molecule to polyethylenglycol, as described in the PCT Patent Application WO 92/13095.

15 The therapeutically effective amount of the active protein(s) will be a function of many variables, including the type of receptor, the affinity of the substance according to the invention to its receptor, any residual cytotoxic activity exhibited thereby, the route of administration, the clinical condition of the patient.

A "therapeutically effective amount" is such that when administered, the
20 substance according to the invention results in a beneficial effect on disease development or progression *in vivo*. The dosage administered, as single or multiple doses, to an individual will vary depending upon a variety of factors, including the pharmacokinetic properties of OPG, the route of administration, patient conditions and characteristics (sex, age, body weight, health, size), extent of symptoms,
25 concurrent treatments, frequency of treatment and the effect desired. Adjustment and manipulation of established dosage ranges are well within the ability of those skilled in the art.

The dose of the polypeptide according to the invention required will vary from about 0,0001 to 100 mg/kg or about 0.01 to 10 mg/kg or about 0.1 to 5 mg/kg or
30 about 1 to 3 mg/kg, although as noted above this will be subject to a great deal of therapeutic discretion. The medicament of the invention may be administered daily, every other day, or three times per week.

The daily doses are usually given in divided doses or in sustained release
35 form effective to obtain the desired results. Second or subsequent administrations can be performed at a dosage, which is the same, less than or greater than the initial or previous dose administered to the individual. A second or subsequent administration can be administered during or prior to onset of the disease.

The invention further relates to a method for treating and/or preventing fibrotic diseases, in particular scleroderma, comprising administering to a patient in need thereof an effective amount of a substance according to the invention, optionally
5 together with a pharmaceutically acceptable carrier. Alternatively, or additionally, a cell producing osteoprotegerin or a nucleic acid molecule of the invention, optionally comprised in an expression vector, may be administered according to the invention.

The expression vector may be administered systemically. Preferably the expression vector is administered by intramuscular injection. A further preferred route
10 of administration is inhalation, in particular if lung fibrosis is involved in the disease. Topical administration of an expression vector comprising OPG sequences, or of an OPG polypeptide according to the invention, is a further preferred route of administration, in particular if there is an involvement of the skin.

The invention further relates to a method for the preparation of a
15 pharmaceutical composition comprising admixing an effective amount of osteoprotegerin with a pharmaceutically acceptable carrier, and to a method of treatment and/or prevention of arthritis comprising administering to a host in need thereof an effective inhibiting amount of osteoprotegerin.

20 All references cited herein, including journal articles or abstracts, published or unpublished U.S. or foreign patent application, issued U.S. or foreign patents or any other references, are entirely incorporated by reference herein, including all data, tables, figures and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by
25 reference.

Reference to known method steps, conventional methods steps, known methods or conventional methods is not any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

30 The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various application such specific embodiments, without undue experimentation, without departing from the general concept of the present invention.
35 Therefore, such adaptations and modifications are intended to be within the meaning an range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology

herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

- 5 Having now described the invention, it will be more readily understood by reference to the following examples that are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLES

10 Methods

Patient samples

Two mm³ punch biopsies were taken from lesional or non-lesional skin (usually from forearm skin) of nine age and sex matched patients with diffuse cutaneous systemic sclerosis (SSc). All patients fulfilled the criteria of the American
15 College of Rheumatology for the diagnosis of SSc.

Fibroblast cultures

Fibroblasts were obtained from the biopsies by *in vitro* culture as previously described (Abraham et al., 1991). Briefly, biopsies were cut into pieces and placed in
20 sterile plastic dishes or flasks. After 15 minutes of drying at room temperature the pieces of biopsy were adherent to the tissue culture plastic and were then cultured in fibroblast growth medium (FGM) consisting of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units per ml penicillin, 100 µg per ml streptomycin, 50 µg per ml
25 gentamicin and 2.5 µg per ml amphotericin B. After 2-3 weeks of incubation in a humidified atmosphere of 5% CO₂ in air, the fibroblast outgrowths were detached by brief trypsinisation and re-cultured in FGM without gentamicin and amphotericin B. In experiments, fibroblasts were used between passages 2 and 5. The fibroblast phenotype was confirmed by their typical morphology in monolayer and three-
30 dimensional collagen gel cultures.

RNA isolation

Total RNA was isolated from confluent scleroderma fibroblasts in early passage using Trizol (Life Technologies) according to the manufacturer's protocol.
35 The final RNA pellet was resuspended in sterile DEPC treated water at a concentration of 1 µg/µl and stored at -80 °C.

cDNA probe synthesis

Two µg total RNA was mixed with 1.3 µl of cytokine specific primers (R&D systems cat. no. GAC11) and incubated at 70 °C for 2 min in a 0.5 ml eppendorf tube. The tubes were then cooled to 50 °C for 2 min after which time reaction mixture containing 2 µl of 5X reaction buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl and 15 mM MgCl₂), 1 µl of 10X dNTP mixture (5 mM dGTP, 5mM dCTP and 5mM dTTP), 3.5 µl of α³²P-dATP (3000Ci/mmol, Amersham cat. no. PB10204), 0.5 µl DTT (100 mM) and 1 µl of superscript II (Life Technologies) were added. The reaction mixture was mixed briefly by pipetting and incubated at 50 °C for 25 min. The reaction was stopped by addition of 1 µl of 0.1 M EDTA pH 8.0 containing 1 mg/ml glycogen). The labeled cDNA was then purified from un-incorporated deoxynucleotides using a Chromaspin-200 DEPC-H₂O column (Clontech) according to the manufacturer's instructions. cDNA containing fractions were identified by Cerenkov counting. The peak fraction (normally fraction 2 out of a total of 6 collected) was treated with 0.1 volumes of 1 M NaOH containing 1 mM EDTA for 20 min at 70 °C to hydrolyze the RNA, and then neutralized with an equal volume of 1 M NaH₂PO₄ containing 2.5 µg of human Cot-1 DNA (Life Technologies) for 20 min at 70 °C. The heat treated neutralized cDNA probe was then added directly to the hybridization mixture.

Hybridization to gene filter microarrays

Gene filter microarrays (human cytokine expression array, R&D systems cat no. GA001) were prehybridized at 68 °C for 2 h in 5 ml of Express Hybridization solution (Clontech) containing 0.5 µg/ml salmon sperm DNA (Life technologies) in roller bottles, in a Hybaid hybridization oven (MWG Biotech). After this time the prehybridization solution was replaced with fresh hybridization solution containing the cDNA probe preparation at a specific activity of between 0.25 to 1 x 10⁶ cpm/ml. Hybridization was for 16-20 h at 68 °C. After hybridization, filters were washed 4 times with 2X SSC/1% SDS at 68 °C for 20 minutes per wash and twice with 0.1X SSC/0.5% SDS for 15 min per wash. Filters were then sealed in Saran wrapTM and exposed to a K-type storage phosphor imaging screen (Biorad) for varying times (4 h to 4 days) at room temperature.

Image analysis

Imaging screens were scanned at a resolution of 50 µm using a Biorad Personal FX phosphorimager. The resultant 16 bit digital file was converted to TIF

format and the image analyzed using Arrayvision software (Imaging Research Inc.) For each sample, we measured the pixel intensity of the 384 genes spotted in duplicate on the filter. The background signal was subtracted and an average pixel intensity for each pair of spots in the array was generated (= expression level).

5

Confirmation of microarray results on selected genes by RT-PCR.

One μg of total RNA from each patient sample was reverse transcribed using an oligo dT primer (Promega) in a 20 μl reaction volume containing 5 mM MgCl_2 , 10 mM Tris-HCl, 50 mM KCl, 0.1 % Triton X-100, 1 mM each of dATP, dGTP, dCTP, dTTP, 0.5 units recombinant RNasin ribonuclease inhibitor (Promega) and 15 units AMV reverse transcriptase (Promega). The reaction mixture was incubated at 42 C for 60 min, heated at 95 C for 5 min then diluted to 200 μl with sterile water. Dilutions of the reverse transcriptase reaction were then subjected to real time PCR analysis on a Taqman (PE Applied Biosystems 7700) using specific primer pairs designed for osteoprotegerin using Primer Express software (PE Applied Biosystems) based on the database accession number given by the gene filter manufacturer: osteoprotegerin-112F 5' CTG CGC GCT CGT GTT TCT and osteoprotegerin-185R 5' AAT GAA GGT ACT TTG GAG GAA ACG. Results were normalized to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in each sample and are expressed as fold changes, determined by dividing the expression value of the abnormal patient sample by the corresponding normal patient sample.

15

20

Cloning of the full cDNA coding sequence of human osteoprotegerin.

The full length cDNA coding sequence of OPG was then cloned by reverse transcriptase PCR using the following primers based on the cDNA sequence available in the EMBL database (accession number U94332): OPG F 5' CGG GAT CCGCCA CCA TGA ACA AGT TGC TGT GCT and OPG R 5' AAG CTC GAG TTA TAA GCA GCT TAT TTT at 50 pmole each in a 50 μl reaction mixture containing 0.3 mM dNTPs, 1 mM MgSO_4 , 5 μl of normal human dermal (foreskin) fibroblast cDNA template (prepared as described above) 5 μl of 10X Pfx amplification buffer (Life Technologies) and 1 μl of Platinum Pfx DNA polymerase (Life Technologies). The reaction mixture was heated at 94 C for 2 min then subjected to 35 cycles of PCR as follows: 94 °C 15 s, 55 C for 30 s and 68 C for 1 min. The amplification products were analyzed on 1 % agarose gels in 1 X TAE buffer (Life Technologies) and PCR

25

30

35

products migrating at the predicted molecular mass (1205 bp) were purified from the gel using the Wizard PCR purification kit (Promega).

One hundred ng of gel-purified DNA was digested with restriction enzymes BamHI and XhoI (Pharmacia), according to the manufacturer's conditions, re-purified as described above and ligated to BamHI/XhoI digested plasmid pcDNA3.1(+) (Invitrogen) using T4 DNA ligase (New England Biolabs) according to standard molecular biology techniques. Ligation products were transformed into *E.coli* strain TOP 10 F' (Invitrogen) by electroporation using a Biorad Gene Pulser. Plasmid DNA was isolated from 5 ml cultures grown up from the resultant colonies and subjected to automated sequence analysis on an Applied Biosystems 3700 sequencer using T7 and pcDNA3.1AS primers (Invitrogen) to confirm the sequence of OPG.

Detection of osteoprotegerin in protein extracts isolated from lesional and normal dermal fibroblasts.

Low passage lesional and normal fibroblasts from scleroderma patients and control subjects, cultured as described above were harvested by treatment of the cell monolayers with PBS containing 1 mM EDTA for 5 min at 37 °C. The detached cells were recovered by centrifugation, resuspended in 50 µl of PBS and treated with 50 µl of sample buffer (20% SDS, 0.2% bromophenol blue, 50% glycerol, 1M Tris pH 6.8), frozen at -80 °C and then thawed. Protein content was determined using a BCA kit (Pierce) according to the manufacturer's protocol. Approximately 10 µg protein was treated with 0.1 volume of DTT (0.1M) and run on 4-12% SDS polyacrylamide gels (Novex) according to the manufacturer's instructions. Protein bands were then transferred to nitrocellulose membranes using a Novex wetblot apparatus at 30 V for 1 h. Membranes were then blocked overnight at 4 °C in PBS containing 5% defatted milk powder then washed in PBST. Membranes were incubated with primary antibody (R&D systems, anti OPG monoclonal antibody, cat no. MAB8051) at 0.5 µg/ml in PBST/1% BSA for 1 h at room temperature with shaking. After this time membranes were washed extensively with PBST prior to incubation with secondary antibody (anti-mouse horseradish peroxidase, Sigma) at a 1/3000 dilution, in PBST/1% BSA. Membranes were finally washed extensively prior to development and visualization, using ECL reagents and Hyperfilm™ from Amersham.

ELISA to measure type I collagen in cell culture supernatants of fibroblasts treated with recombinant OPG or following transfection of OPG/pcDNA3.1 vector

Collagen synthesis was measured *in vitro* in cultured human fibroblasts using a capture ELISA system as described by Shi-wen et al., (Shi-Wen et al., 1997):

5 Briefly, AG1518 human dermal fibroblasts (purchased from ATCC) were maintained in DMEM medium containing 5% FCS and 2 mM glutamine (Life Technologies). Cells were harvested by trypsinization and seeded at 70% confluence in 48 well cell culture plates (Falcon) in medium containing 5 % serum, then transferred to serum free medium 8 h later. After 24 h, medium was removed and replaced with fresh

10 serum free medium containing 50 μ M L-ascorbate (Wako Pure Chemical Industries) and increasing concentrations of human recombinant osteoprotegerin (OPG-Fc, purchased from R&D systems). Sixteen hours later, TGF β 1 (PeproTech) was added to the medium to a final concentration of 2 ng/ml, and after a further 24 h, the medium was recovered, centrifuged to remove cell debris, diluted, and subjected to

15 ELISA for type I collagen as described below.

Collagen synthesis was also measured in primary human fibroblasts (OBHC cells) following transfection with OPG cDNA (pcDNA3.1-OPG) or after mock transfection (pcDNA3.1 vector alone) as follows: OBHC cells were harvested by trypsinization and plated at 50% confluence in 48 well plates in complete medium.

20 Plasmids were transfected into OBHC cells using the Eugene V transfection reagent (Roche) according to the manufacturer's instructions. Five hours after transfection, medium was replaced with fresh serum free medium containing L-ascorbate overnight, then treated with TGF β 1 (10 ng/ml) for 24 h as described above.

MaxiSorp plates (Nunc) were coated overnight, at 4°C with 5 μ g/ml goat anti-human type-I collagen (Southern Biotechnology Associates, Inc.) in PBS (pH 7.4). The antibody solution was then removed and plates were blocked with PBS-1% BSA for 1 h at room temperature. Plates were then washed 4 times PBS/0.05% Tween 20 (PBST). Triplicate samples of 100 μ l conditioned media were tested. Samples were

25 incubated for 2 h at room temperature (RT) then washed 4X with PBST. Samples were then incubated with a 1:2000 dilution of biotinylated goat anti-human type-I collagen antibody (Southern Biotechnology Associates, Inc.) for 1 h at RT then washed 4X with PBST. Samples were then incubated with a 1/4000 dilution of HRP-streptavidin conjugated antibody (Zymed) for 1 h at RT with shaking. Finally,

30 samples were washed 4X with PBST. OPD substrate (Sigma cat. no. P9187) was added and left for 10 minutes for colour development. The reaction was stopped with

35 20% H₂SO₄ mix and plates read at 496 nm on a Victor² multilabel counter (Wallac).

Results were normalized for cell numbers using CyQuant cell proliferation assay kit (C-7026) from Molecular Probes.

In vivo studies

5 Lung fibrosis was induced in male mice (20–25 g) by intra-tracheal administration of bleomycin (0.075 IU) in saline on day 1. Mice were divided into 3 separate groups of 10 animals. One group received sub cutaneous (s.c) injections of OPG at 5 mg/kg (in saline) daily. A second group received OPG (0.5 mg/kg) s.c. daily. The third group received saline (s.c.) daily. A fourth group comprised age and sex matched untreated naïve mice. Body mass was measured daily and all mice
10 were sacrificed on day 12. Individual lung lobes were isolated for hydroxypyrrolone determination (Smith et al., 1994), or were formalin fixed and paraffin embedded for histology. Lung sections were stained with tri-chrome or picro sirius red for collagen (Bancroft and Stevens) and the extent of fibrosis/lung was scored.

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*Semi-quantitative reverse transcriptase PCR analysis of OPG mRNA in scleroderma patient fibroblasts treated *in vitro* with halofuginone.*

Lesional and non-lesional fibroblasts were maintained in culture as described above. Cell monolayers (70% confluent) were treated with halofuginone (10^{-8} M)
20 (Collgard Pharmaceuticals) for 12 h. After the treatment period, cells were harvested and total RNA was isolated using the Trizol method. One μ g of total RNA was then subjected to reverse transcriptase PCR as described above using PCR primers specific for osteoprotegerin (OPG 740 F 5' ACG CCT AAC TGG CTT AGT GT and OPG 1280R 5' CTG ATT GGA CCT GGT TAC CT). After 30 cycles of PCR, reaction
25 products were run on 1% agarose gels stained with ethidium bromide and photographed and analysed using Kodak 1D Digital Science software. PCR products migrating at the predicted molecular weight were then verified to be osteoprotegerin by direct sequencing of the gel extracted DNA. As a control for genomic DNA contamination, PCR reactions were performed on reverse transcriptase reaction
30 mixes which contained the appropriate RNA but no reverse transcriptase. Reverse transcriptase reactions were also amplified with GAPDH specific PCR primers as a positive control for the integrity and quantity of input RNA in the initial reaction.

Type I collagen α 2 promoter activity.

35 Mouse embryo fibroblasts (π S3 cells) were maintained in DMEM-F12 medium containing 2% FCS and 2 mM glutamine. Cells were harvested by trypsinization and

plated at 50% confluence in 48 well cell culture plates. The next day cells were transfected in serum free medium with pGL3 vector (Promega) containing 3.5 kb of collagen 1 α 2 promoter linked to luciferase cDNA (kindly provided by Dr. David Abraham from the Royal Free Hospital, London, England) using the Eugene V reagent (Roche) according to the manufacturer's instruction. After 5 h transfection, cells were transferred to fresh medium containing 2% FCS and treated with halofuginone alone (10^{-10} M), TGF β 1 (5 ng/ml) alone or HF (10^{-8} M) + TGF β 1 (5 ng/ml) for 12 h in the presence of 0, 1, 10 or 100 ng/ml of recombinant human osteoprotegerin. After the treatment period, cells were transferred to complete medium containing 2 % FCS and 2 mM glutamine and luciferase activity was measured 48 h later in each well, using the Bright-Glo assay system purchased from Promega. Results are expressed as relative luminescence units and were normalized to the number of cells/well determined in a parallel experiment using the Cyquant kit (Molecular Probes).

Example 1: osteoprotegerin mRNA is significantly down regulated in lesional versus non-lesional fibroblasts

Gene filter microarray analysis was performed on normal and abnormal fibroblast samples from 6 scleroderma patients. The mean expression level of osteoprotegerin (OPG) is shown in Fig. 1a and for each patient in Fig. 1b.

The results obtained on the microarrays were further corroborated by real time PCR analysis of RNA samples isolated from 9 patients using OPG specific PCR primers. Results are shown in Fig. 2 and are expressed as the fold change in expression level (lesional divided by non-lesional). In 7 out of the 9 patients tested, OPG was downregulated at least 2 fold in lesional fibroblasts compared to non-lesional fibroblasts isolated from the same patient.

The differences in expression observed between non-lesional and lesional fibroblasts from the same patient are unlikely to be due differences in the culture conditions between the two populations since OPG mRNA expression in primary cultures of normal human dermal fibroblasts does not change significantly on passaging the cells (data not shown). In addition real time RT-PCR analysis of total RNA isolated from whole biopsy specimens of abnormal skin from scleroderma patients indicated lower levels of osteoprotegerin mRNA compared to clinically normal looking anatomical site matched skin, from the same patients (Figure 3).

Example 2: OPG is downregulated at the protein level in lesional vs. non lesional fibroblasts from scleroderma patients

In order to determine if the downregulation of osteoprotegerin mRNA reflected a change in osteoprotegerin protein, the OPG content of anatomical site matched normal and lesional skin fibroblasts from age and sex matched subjects was determined by Western Blot analysis using anti-human osteoprotegerin monoclonal antibodies. Results are shown in Figure 4. Osteoprotegrin monomer and dimer were clearly detectable in 3/4 normal fibroblasts and weakly expressed in 1 normal sample. In the abnormal fibroblasts a monomeric band was weakly visible in all four samples tested. Recombinantly expressed OPG-Fc fusion protein served as positive control for the staining.

Example 3: Cloning of human OPG

In order to characterise OPG activity *in vitro* and *in vivo* the full cDNA coding sequence was cloned by reverse transcriptase PCR using primers based on the published sequence of OPG, which flanked the predicted start and stop codons. Sequence analysis of the resultant OPG cDNA clones (in pcDNA3.1) revealed 100% identity at the nucleotide level to sequence of OPG published by Morinaga et al., (Morianga et al., 1998) but differed by 1 amino acid to the sequence published by Simonet et al. (1997) (see SEQ ID NO: 2 and 4).

Example 4: Effect on OPG type I collagen synthesis in a human dermal fibroblast cell line

Fibroblasts cultured in the presence of L-ascorbate secrete collagen into the culture medium allowing collagen detection by ELISA. Human fibroblasts upregulate collagen synthesis in response to stimulation with the profibrotic cytokine TGF β 1. We therefore tested the effect of recombinant osteoprotegerin (osteoprotegerin-Fc fusion protein) on TGF β 1 induced collagen synthesis in AG1518C fibroblasts. osteoprotegerin added to fibroblast cultures prior to TGF β 1 treatment was able to inhibit the TGF β 1 mediated increase in collagen synthesis in a dose dependent manner (Figure 5). When the experiment was performed in the presence of neutralizing anti OPG monoclonal antibodies there was no effect on collagen synthesis. Similarly, administration of anti OPG alone in the absence of recombinant OPG had no effect on collagen synthesis suggesting that the decrease in collagen synthesis observed was a specific effect of OPG.

We observed a similar effect on collagen synthesis upon transfection of primary human fibroblasts (OBHC) with a plasmid containing the full coding sequence of OPG (pcDNA3.1/OPG). In OBHC transfected with OPG plasmid, no increase in collagen synthesis was observed in response to TGF β 1 stimulation, in contrast to mock (pcDNA3.1 empty vector) transfected OBHC (Figure 6).

Example 5: OPG treatment significantly decreases both basal and TGF β 1 induced type 1 collagen alpha 2 promoter activity in mouse embryo fibroblasts

In order to evaluate if the decrease in collagen synthesis by OPG was due to an effect on collagen promoter activity we examined the effect of OPG treatment on mouse embryo fibroblasts which had been transfected with a plasmid which contained a reporter gene cDNA, luciferase, under the control of a 3.5 kb region of the type I collagen α 2 promoter. Stimulation of the collagen promoter in this construct leads to transcriptional activation of the luciferase gene whose activity can be measured in the presence of its substrate firefly luciferin by luminescence assay.

Treatment with OPG from 1 ng/ml to 100 ng/ml was able to significantly decrease the basal level of collagen promoter activity (Figure 7, control). Following TGF β 1 stimulation there was at least a 2 fold increase in collagen promoter activity (Figure 7, TGF β 1). This could be significantly decreased when the cells were concurrently treated with OPG at 100 ng/ml ($P \leq 0.05$).

The plant alkaloid halofuginone has been reported to be a specific inhibitor of type I collagen synthesis (Granot et al., 1993). When fibroblasts were treated with low concentrations of halofuginone (10^{-10} M) we observed a dose dependent decrease in collagen promoter activity with increasing concentrations of osteoprotegerin (from 1 ng/ml to 100 ng/ml) which was highly significant at the highest dose of OPG tested (100 ng/ml, $P \leq 0.01$) (Figure 7, HF). Halofuginone can inhibit TGF β 1 induced collagen promoter activity (McGaha et al 2002). OPG was also able to potentiate the effects of halofuginone by inhibiting the TGF β 1 induced increase in collagen promoter activity in a dose dependent manner. This effect was highly significant at 100 ng/ml OPG ($P \leq 0.01$) at which concentration the collagen promoter activity was almost returned to basal levels (Figure 7, HF+TGF β 1).

Example 6: Expression of OPG mRNA in halofuginone treated scleroderma patient fibroblasts

The mechanism by which halofuginone is able to down regulate collagen synthesis in fibroblasts has not been fully elucidated. We therefore examined the

effect of halofuginone on the expression of OPG mRNA in paired lesional and non-lesional fibroblasts from scleroderma patients by reverse transcriptase PCR.

OPG mRNA expression is highly upregulated (at least 3 fold) in both lesional and non lesional fibroblasts in all samples tested (3 normals and 2 abnormal) when measured 12 h after treatment with 10^{-8} M halofuginone (Figure 8). Interestingly, in gene filter microarray analysis experiments OPG was one of the most upregulated genes on treatment of fibroblasts with halofuginone (data not shown).

Example 7: OPG administration *in vivo* protects against bleomycin induced lung fibrosis in mice

Administration of a single intra-tracheal injection bleomycin to C57BL/6 mice results in the rapid induction of pulmonary fibrosis within 14 days, which is characterized by increased collagen deposition within the lung interstitium (Hattori et al., 2000). In order to determine if OPG administration could have any protective effect against the development of fibrosis or indeed reduce the severity of the disease, we tested the effect of daily injection of OPG in bleomycin treated mice.

Bleomycin was administered by a single intra-tracheal instillation to 3 groups of 10 mice. OPG was administered by subcutaneous injection starting the day after bleomycin treatment. One group received a high dose (5 mg/kg) and the second group received a lower dose (0.5 mg/kg). Control mice received saline s.c. daily. For comparison a 4th group of mice was included in the study which comprised completely untreated, age and sex matched individuals (naïve mice).

Animals treated with bleomycin fall sick immediately. This results in a rapid loss of body mass and can lead to death, in contrast to untreated animals which remain happy and healthy and show normal weight gain (Figure 9A). Mice treated with the low dose of OPG showed a comparable weight loss to the control animals. There also appeared to be increased mortality in this group (Figure 9B). Mice receiving the high dose of OPG fared much better, with a decline in body mass only for the first 5 days of treatment. This also reflected the low mortality (<10%) in this group.

All surviving mice were sacrificed 12 days after the bleomycin treatment. Histological analysis of lungs revealed that in the high dose OPG treated animals there was significantly less lung surface area affected by fibrosis compared to control animals which had fibrotic lesions covering approx. 70% of the lung surface (Fig. 10A). High dose OPG treated mice also showed significantly less hydroxyproline content in the lungs ($P \leq 0.05$) compared to control mice and was comparable to that seen in the untreated mice (naïve group, Fig. 10 B)). Reduced

hydroxyproline content was also observed in the low dose OPG treated mice, although this did not reach statistical significance due to the small number of survivors left in this group. Hydroxyproline content is a measure of collagen deposition. The results here indicate that OPG treatment effectively leads to reduced collagen deposition in the lungs.

REFERENCES

1. Abraham D., Lupoli S., McWhirter A., Plater-Zyberk C., Piela T.H., Korn J.H., Olsen I. and Black C. (1991) Expression and function of surface antigens on scleroderma fibroblasts. *Arthritis Rheum.* 34, 1164-1172.
- 5 2. Abraham DJ, Shiwen X, Black CM, Sa S, Xu Y, Leask A.J *Biol Chem.* 2000 May 19;275(20):15220-5.
3. Altschul S F et al, *J Mol Biol*, 215, 403-410, 1990
4. Altschul S F et al, *Nucleic Acids Res.*, 25:389-3402, 1997
- 10 5. Bancroft J.D. and Stevens A. *Theory and Practice of Histological Techniques.* Churchill Livingstone, England.
6. Black C.M and Denton C.P. (1998) Systemic sclerosis and related disorders. In "Oxford textbook of Rheumatology" (P.G. Maddison, D.A. Isenberg, P.Woo and D.N. Glass, Eds.) pp 771-789, Oxford Univ. Press, New York.
- 15 7. Clements P.J. and Furst D.E. (1996) "Systemic Sclerosis" Williams and Williams, Baltimore.
8. Devereux J et al, *Nucleic Acids Res*, 12, 387-395, 1984.
9. Engelmann, H., Novick, D., and Wallach, D., 1990, *J.Biol.Chem.* 265, 1531-1536.
- 20 10. Granot I, Halevy O, Hurwitz S, Pines M. (1993) Halofuginone: an inhibitor of collagen type I synthesis. *Biochim Biophys Acta* 1156, 107-112
11. Grantham (1974), *Science*, 185. 862-864.
12. Hattori N., Degen J.L., Sisson T.H., Liu H., Moore B.B., Pandrangi R.G., Simon R.H. and Drew A.F. (2000) Bleomycin induced pulmonary fibrosis in fibrinogen null mice. *J. Clin. Invest.* 106, 1341-135
- 25 13. Kostenuik PJ, Shalhoub V. *Curr Pharm Des* 2001 May;7(8):613-35
14. Krein, PM, Huang Y and Winston BW (2001). *Expert Opin. Ther. Patents* 11(7): 1065-1079.
15. Leighton, C. *Drugs* 2001 61(3), 419-427.
- 30 16. LeRoy E.C. (1974) Increased collagen synthesis by scleroderma skin fibroblasts in vitro. *J. Clin. Invest.* 54, 880-889.
17. Martini, Maccario, Ravelli et al., *Arthritis Rheum.* 1999, 42, 807-811.
18. McGaha TL, Phelps RG, Spiera H, Bona C. (2002) Halofuginone, an Inhibitor of Type-I Collagen Synthesis and Skin Sclerosis, Blocks Transforming-Growth-Factor-beta-Mediated Smad3 Activation in Fibroblasts. *J Invest Dermatol.* 118,461-70.
- 35

19. Min H., Morony S., Sarosi I., Dunstan C.R., Capparelli C. et al (2000).
Osteoprotegerin reverses osteoporosis by inhibiting endosteal osteoclasts
and prevents vascular calcification by blocking a process resembling
osteoclastogenesis. *J.Exp. Med.* 192, 463-474.
- 5 20. Morinaga T., Nagakawa N., Yasuda H., Tsuda E. and Higashi K. (1998)
Cloning and characterization of the gene encoding human osteoprotegerin/
osteoclastogenesis inhibitory factor. *Eur. J. Biochem.* 254, 685-691.
21. Pearson W R, *Methods in Enzymology*, 183, 63-99, 1990
22. Pearson W R and Lipman D J, *Proc Nat Acad Sci USA*, 85, 2444 -2448, 1988
- 10 23. Silman A.J. (1991) Moratlity from scleroderma in England and Wales 1968 -
1975. *Ann. Rheu. Dis.* 50, 95-96.
24. Simonet W.S., Lacey D., Dunstan C.R., Kelley M., et al. (1997)
Osteoprotegerin: a novel secreted protein involved in the regulation of bone
densit y. *Cell* 89, 309-319.
- 15 25. Shi-wen X., Denton C.P., McWhirter A., Bou-Gharios G., Abraham D.J., du
Bois R.M. and Black C.M. (1997) Scleroderma lung fibroblasts exhibit
elevated and dysregulated collagen type I biosynthesis. *Arthritis Rheum.* 40,
1237-1244.
- 20 26. Shi-Wen X, Denton CP, McWhirter A, Bou-Gharios G, Abraham DJ, du Bois
RM, Black CM. (1997) Scleroderma lung fibroblasts exhibit elevated and
dysregulated type I collagen biosynthesis. *Arthritis Rheum.* 40, 12 37-1244
27. Smith and Waterman *J Mol Biol*, 147,195-197, 1981, *Advances in Applied*
Mathematics, 2, 482-489, 1981.
- 25 28. Smith R.E., Strieter R.M., Phan S.H., Lukacs N.W., Huffnagle G.B., Wilke
C.A., Burdick M.D., Lincoln P., Evanoff H. and Kunkel S.L. (1994) Producti on
and function of murine macrophage inflammatory protein -1 α in bleomycin
induced lung injury. *J. Immunol.* 153, 4704.
29. Smith, *Textbook of the Autoimmune Diseases*, Edited by Lahita, Chiorazzi
and Reeves, Lippincott Williams & Wilkins, Philadelphia 2000.
- 30 30. Strehlow D. and Korn J (1998) Biology of the scleroderma fibroblast. *Curr.*
Opin. Rheumatol. 10, 572-578.
31. Tucci, A., James, H., Chicheportiche, R., Bonnefoy, J.Y., Dayer, J.M., and
Zubler, R.H., 1992, *J.Immunol.* 148, 2778-2784.
32. Von Heijne G. (1986) *Nucleic Acids Res.* 14, 4683-4690.
- 35 33. Wigley F. M. and Sule S. D. (2001) Expert Opinions on Investigational Drugs
10(1) 31-48.

34. Wigley F.M. and Boling C.L. (2000) The treatment of scleroderma. 2, 276-292.
35. Wilm M., Shevchenko A., Houthaeve T., Breit S., Schweigerer L., Fotsis T. and Mann M. (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature*, 379:466-469
36. Yasuda H., Shima N., Nagakawa N., Mochizuki S., Yano K. et al (1998) Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis in vitro. *Endocrinology* 139, 1329-1337.

CLAIMS

1. Use of a substance for the manufacture of a medicament for the treatment and/or prevention of a fibrotic disease, wherein the substance is selected from the group consisting of:
 - 5 a) A polypeptide comprising SEQ ID NO: 2 or SEQ ID NO: 4;
 - b) A polypeptide comprising amino acids 22 to 401 of SEQ ID NO: 2 or SEQ ID NO: 4;
 - c) A polypeptide comprising one, two, three or four cysteine-rich domains of osteoprotegerin;
 - 10 d) A polypeptide comprising amino acids 22 to 194 of SEQ ID NO: 2 or SEQ ID NO: 4;
 - e) A mutein of any of (a) to (d), wherein the amino acid sequence has at least 40 % or 50 % or 60 % or 70 % or 80 % or 90 % identity to at least one of the sequences in (a) to (d);
 - 15 f) A mutein of any of (a) to (d) which is encoded by a DNA sequence which hybridizes to the complement of the DNA sequence encoding any of (a) to (d) under moderately stringent conditions or under high stringent conditions;
 - g) A mutein of any of (a) to (d) wherein any changes in the amino acid sequence are conservative amino acid substitutions to the amino acid sequences in (a) to (d);
 - 20 h) a salt or an isoform, fused protein, functional derivative, active fraction or circularly permuted derivative of any of (a) to (g).
- 25 2. Use according to claim 1, wherein the fibrotic disease is a connective tissue disease.
3. Use according to claim 1 or 2, wherein the fibrotic disease is scleroderma.
- 30 4. Use according to any preceding claims, wherein the substance is a monomer or dimer.
5. Use according to any preceding claims, wherein the substance is glycosylated at one or more sites.
- 35 6. Use according to any of the preceding claims, wherein the fused protein comprises an immunoglobulin (Ig) fusion.

7. Use according to claim 6, wherein the Ig fusion is an Fc fusion.
8. Use according to any of the preceding claims, wherein the functional derivative
5 comprises at least one moiety attached to one or more functional groups, which
occur as one or more side chains on the amino acid residues.
9. Use according to claim 8, wherein the moiety is a polyethylene moiety.
10. Use of a nucleic acid molecule for manufacture of a medicament for the treatment
10 and/or prevention of a fibrotic disease, wherein the nucleic acid molecule
comprises a nucleic acid sequence encoding a polypeptide comprising an amino
acid sequence selected from the group consisting of:
- a) A polypeptide comprising SEQ ID NO: 2 or SEQ ID NO: 4;
 - 15 b) A polypeptide comprising amino acids 22 to 401 of SEQ ID NO: 2 or SEQ
ID NO: 4;
 - c) A polypeptide comprising one, two, three or four cystein-rich domains of
osteoprotegerin;
 - d) A polypeptide comprising amino acids 22 to 194 of SEQ ID NO: 2 SEQ ID
20 NO: 4;
 - e) A mutein of any of (a) to (d), wherein the amino acid sequence has at
least 40 % or 50 % or 60 % or 70 % or 80 % or 90 % identity to at least
one of the sequences in (a) to (d);
 - 25 f) A mutein of any of (a) to (d) which is encoded by a DNA sequence which
hybridizes to the complement of the native DNA sequence encoding any
of (a) to (d) under moderately stringent conditions or under high stringent
conditions;
 - g) A mutein of any of (a) to (d) wherein any changes in the amino acid
30 sequence are conservative amino acid substitutions to the amino acid
sequences in (a) to (d);
 - h) An isoform, fused protein or active fraction of any of (a) to (g).
11. Use according to claim 10, wherein the fibrotic disease is a connective tissue
35 disease.
12. Use according to claim 10 or 11, wherein the fibrotic disease is scleroderma.

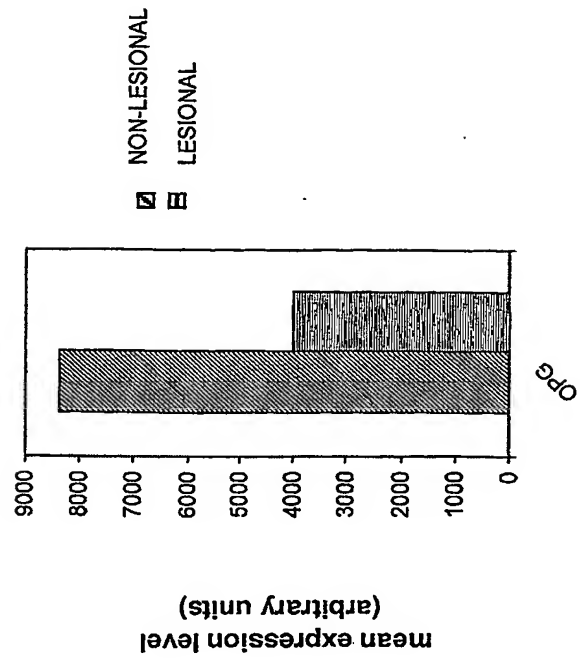
13. Use according to any of claims 10 to 12, wherein the nucleic acid molecule comprises an expression vector sequence.
- 5 14. Use according to claim 13, wherein the vector sequence is a gene therapy vector sequence.
- 10 15. Use of a vector for inducing and/or enhancing the endogenous production of a polypeptide according to claim 1 in a cell for the preparation of a medicament for the treatment and/or prevention of a fibrotic disease, in particular scleroderma.
- 15 16. Use of a cell comprising a nucleic acid molecule according to any of claims 10 to 15 for the preparation of a medicament for the treatment and/or prevention of fibrotic disease, in particular scleroderma.
- 20 17. Use of a cell expressing a substance according to claim 1 to 9 for the manufacture of a medicament for the treatment and/or prevention of a fibrotic disease, in particular scleroderma.
- 25 18. Use of a cell that has been genetically modified to produce a polypeptide according claim 1 or 4 to 9 for the manufacture of a medicament for the treatment and/or prevention of a fibrotic disease, in particular scleroderma.
- 30 19. The use according to any of the preceding claims, wherein the medicament further comprises an interferon, for simultaneous, sequential, or separate use.
20. The use according to claim 19, wherein the interferon is interferon- β .
21. The use according to any of the preceding claims, wherein the medicament further comprises a Tumor Necrosis Factor (TNF) antagonist for simultaneous, sequential, or separate use.
22. The use according to claim 21, wherein the TNF antagonist is TBPI and/or TBPII.
23. The use according to any of the preceding claims, wherein the medicament further comprises an anti-scleroderma agent for simultaneous, sequential, or separate use.

24. The use according to claim 25, wherein the anti-scleroderma agent is selected from the group consisting of halofuginone, ACE inhibitors, calcium channel blockers, proton pump inhibitors, NSAIDs, COX-inhibitors, corticosteroids, tetracycline, pentoxifylline, bucillamine, geranylgeranyl transferase inhibitors, rotterlin, prolyl-4-hydroxylase inhibitors, c-proteinase inhibitors, lysyl-oxidase inhibitors, relaxin, halofuginone, prostaglandins, prostacyclins, endothelin-1, nitric oxide, angiotensin II inhibitors and anti-oxidants.
25. Method for treating and/or preventing a fibrotic disease, in particular scleroderma, comprising administering to a patient in need thereof an effective amount of a substance according to any of claims 1 to 18, optionally together with a pharmaceutically acceptable carrier.

ABSTRACT

The invention relates to the use of osteoprotegerin for treatment and/or prevention of fibrotic diseases, in particular of scleroderma.

a



b

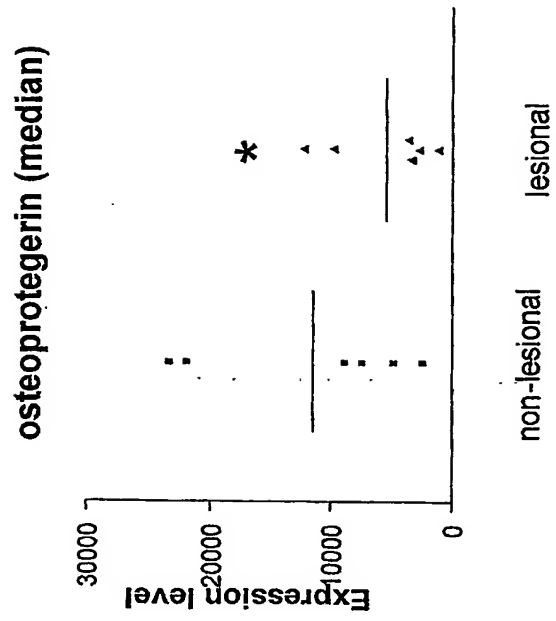


Fig. 1

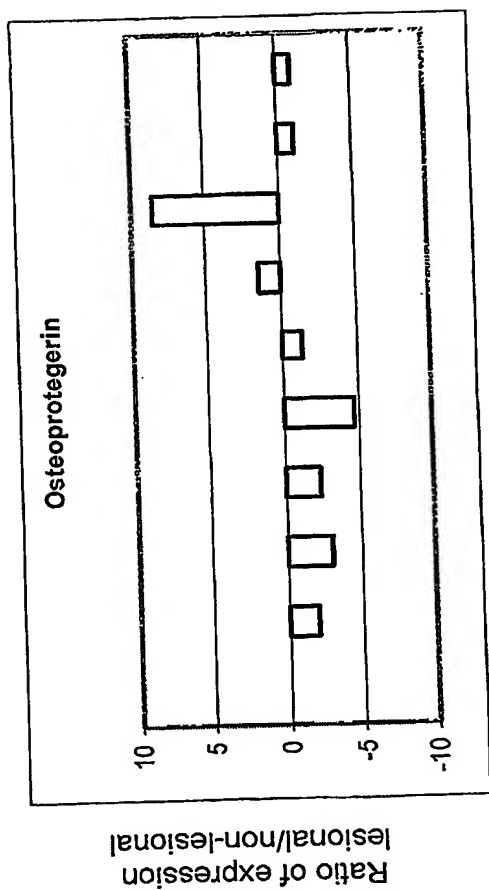


Fig. 2

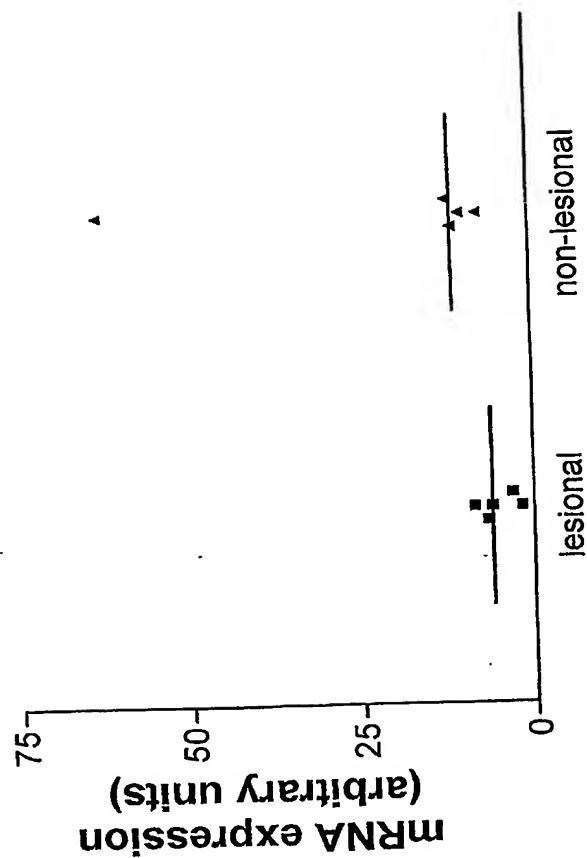


Fig. 3

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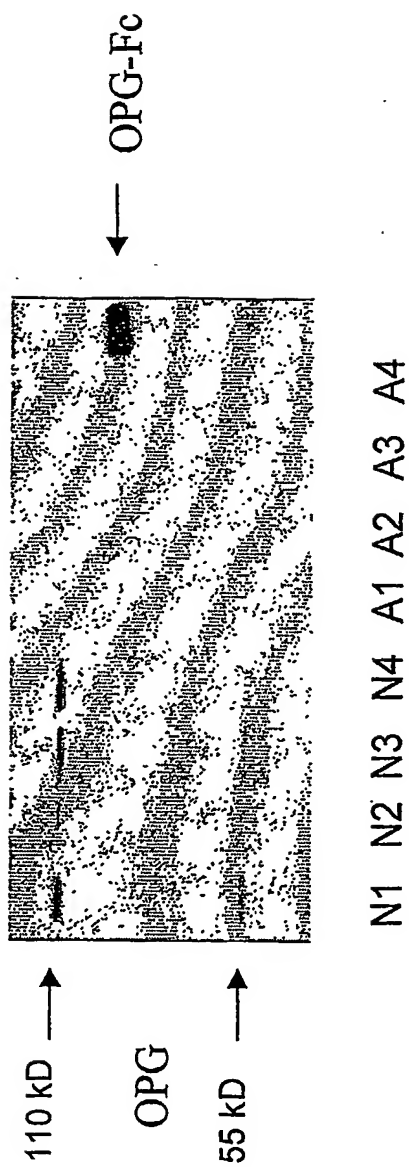


Fig. 4

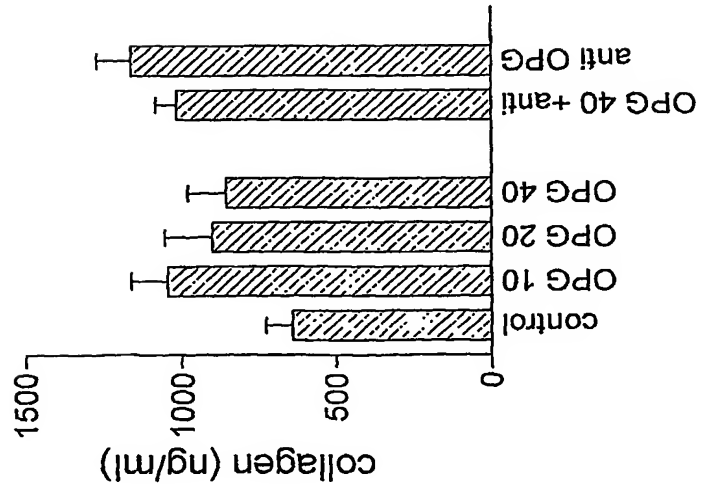


Fig. 5

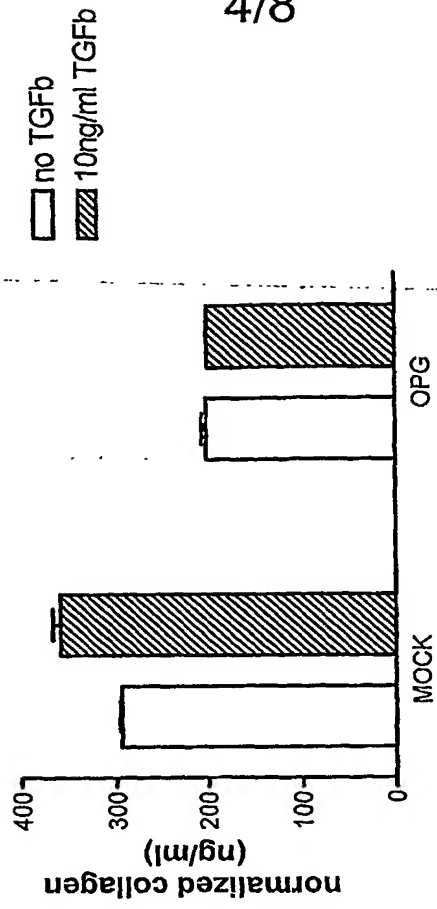


Fig. 6

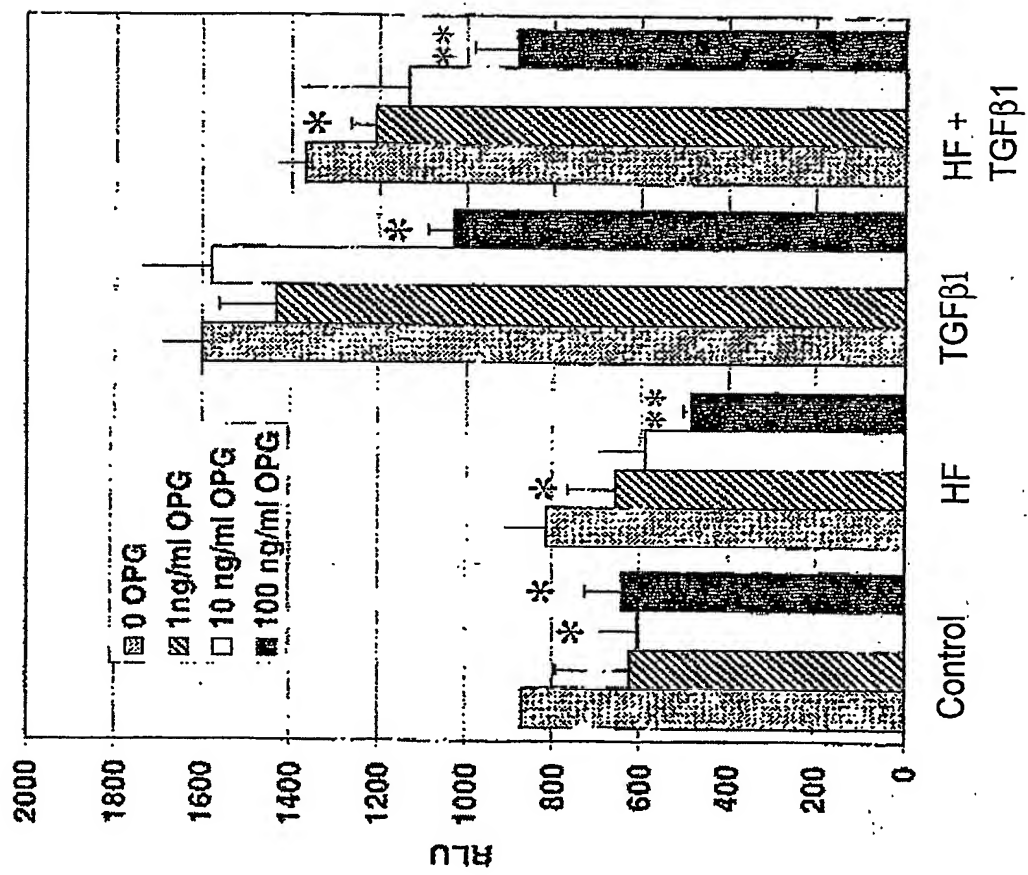


Fig. 7

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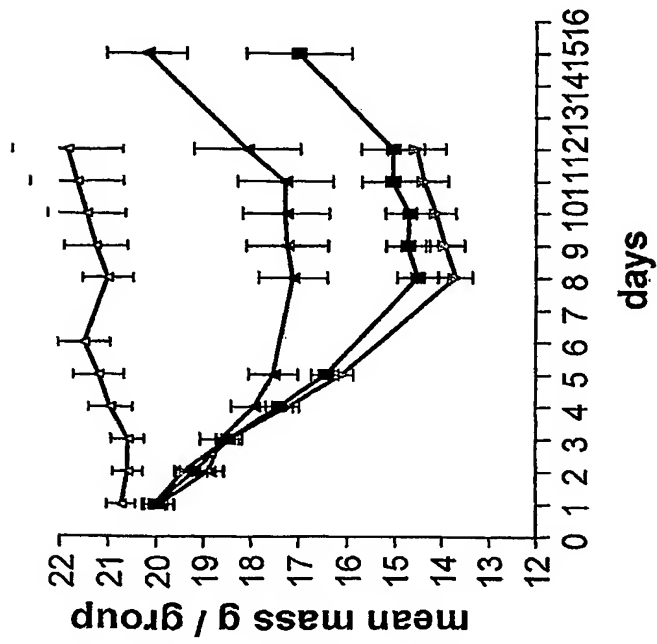


Osteoprotegerin

GAPDH

Fig. 8

A



B

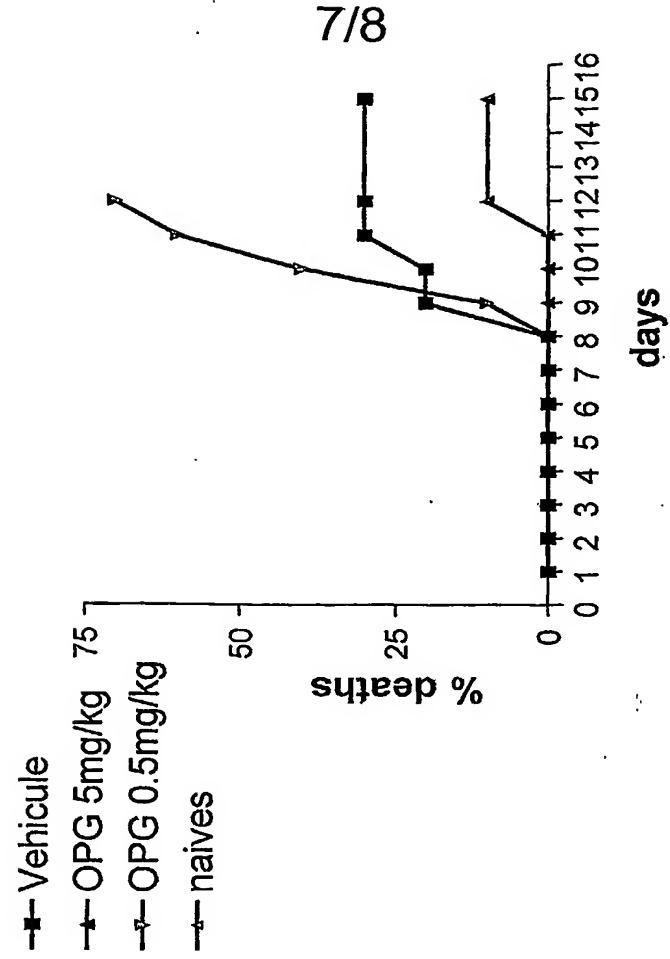


Fig. 9

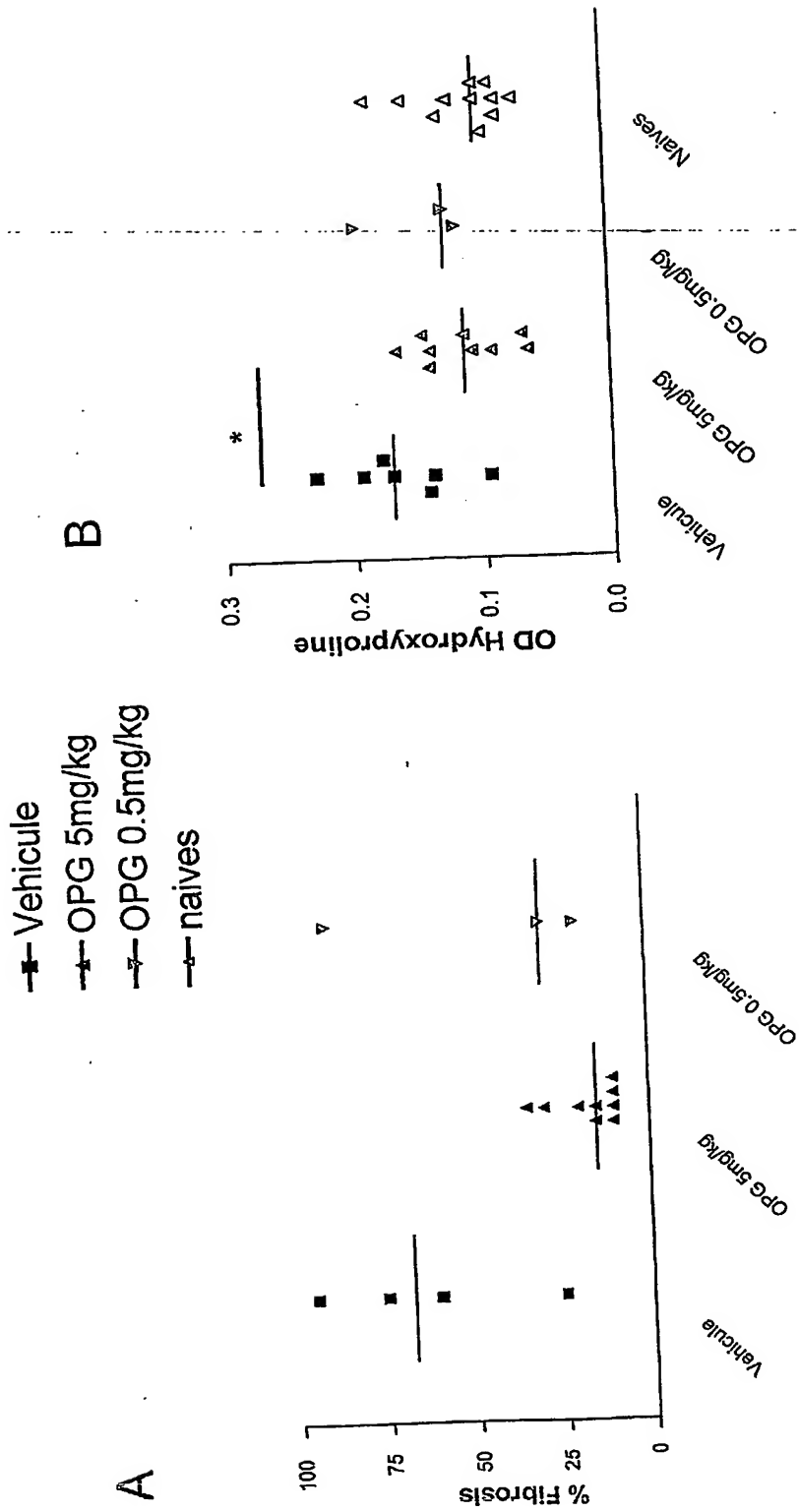


Fig. 10

SEQUENCE LISTING

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| tctcccaacc tcccagcgga ccggcgggga gaaggctcca ctcgctccct ccagggagag | 1320 |

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Lys Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp

20 25 30

Ile Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr

35 40 45

Tyr Leu Lys Gln His Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro

50

55

60

Cys Pro Asp His Tyr Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys

65

70

75

80

Leu Tyr Cys Ser Pro Val Cys Lys Glu Leu Gln Tyr Val Lys Gln Glu

85

90

95

Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr

100

105

110

Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe

115

120

125

Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg

130

135

140

Cys Pro Asp Gly Phe Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys

145

150

155

160

Arg Lys His Thr Asn Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys

165

170

175

Gly Asn Ala Thr His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr

180

185

190

Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg

195

200

205

Phe Ala Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val

210

215

220

Asp Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile

225

230

235

240

Lys Arg Gln His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu

245

250

255

Trp Lys His Gln Asn Lys Asp Gln Asp Ile Val Lys Lys Ile Ile Gln

260

265

270

Asp Ile Asp Leu Cys Glu Asn Ser Val Gln Arg His Ile Gly His Ala

275

280

285

Asn Leu Thr Phe Glu Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly

290

295

300

Lys Lys Val Gly Ala Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys

305

310

315

320

Pro Ser Asp Gln Ile Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn

325

330

335

Gly Asp Gln Asp Thr Leu Lys Gly Leu Met His Ala Leu Lys His Ser
340 345 350

Lys Thr Tyr His Phe Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr
355 360 365

Ile Arg Phe Leu His Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu
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Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys
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Leu

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<212> DNA

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ggcgctccac gacatctc ccctgggac caccctgtt ccttctctcc caccgctcc 180

| | |
|---|------|
| cattatgac gaagaaacct ctcatcagct gttgtgtgac aaatgtcctc ctggtaccta | 240 |
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| ctacacagac agctggcaca ccagtgcga gtgtctatac tgcagccccg tgtgcaagga | 360 |
| gtgcagtac gtcaagcagg agtgcaatcg caccacaac cgcgtgtgcg aatgcaagga | 420 |
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<213> Homo sapiens

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Met Asn Lys Leu Leu Cys Cys Ala Leu Val Phe Leu Asp Ile Ser Ile

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Lys Trp Thr Thr Gln Glu Thr Phe Pro Pro Lys Tyr Leu His Tyr Asp

20 25 30

Glu Glu Thr Ser His Gln Leu Leu Cys Asp Lys Cys Pro Pro Gly Thr

35 40 45

Tyr Leu Lys Gln His Cys Thr Ala Lys Trp Lys Thr Val Cys Ala Pro

50 55 60

Cys Pro Asp His Tyr Tyr Thr Asp Ser Trp His Thr Ser Asp Glu Cys

65 70 75 80

Leu Tyr Cys Ser Pro Val Lys Glu Leu Gln Tyr Val Lys Gln

85

90

95

Cys Asn Arg Thr His Asn Arg Val Cys Glu Cys Lys Glu Gly Arg Tyr

100

105

110

Leu Glu Ile Glu Phe Cys Leu Lys His Arg Ser Cys Pro Pro Gly Phe

115

120

125

Gly Val Val Gln Ala Gly Thr Pro Glu Arg Asn Thr Val Cys Lys Arg

130

135

140

Cys Pro Asp Gly Phe Phe Ser Asn Glu Thr Ser Ser Lys Ala Pro Cys

145

150

155

160

Arg Lys His Thr Asn Cys Ser Val Phe Gly Leu Leu Leu Thr Gln Lys

165

170

175

Gly Asn Ala Thr His Asp Asn Ile Cys Ser Gly Asn Ser Glu Ser Thr

180

185

190

Gln Lys Cys Gly Ile Asp Val Thr Leu Cys Glu Glu Ala Phe Phe Arg

195

200

205

Phe Ala Val Pro Thr Lys Phe Thr Pro Asn Trp Leu Ser Val Leu Val

210

215

220

Asp Asn Leu Pro Gly Thr Lys Val Asn Ala Glu Ser Val Glu Arg Ile

225

230

235

240

Lys Arg Gln His Ser Ser Gln Glu Gln Thr Phe Gln Leu Leu Lys Leu

245

250

255

Trp Lys His Gln Asn Lys Ala Gln Asp Ile Val Lys Lys Ile Ile Gln

260

265

270

Asp Ile Asp Leu Cys Glu Asn Ser Val Gln Arg His Ile Gly His Ala

275

280

285

Asn Leu Thr Phe Glu Gln Leu Arg Ser Leu Met Glu Ser Leu Pro Gly

290

295

300

Lys Lys Val Gly Ala Glu Asp Ile Glu Lys Thr Ile Lys Ala Cys Lys

305

310

315

320

Pro Ser Asp Gln Ile Leu Lys Leu Leu Ser Leu Trp Arg Ile Lys Asn

325

330

335

Gly Asp Gln Asp Thr Leu Lys Gly Leu Met His Ala Leu Lys His Ser

340

345

350

Lys Thr Tyr His Phe Pro Lys Thr Val Thr Gln Ser Leu Lys Lys Thr

355

360

365

Ile Arg Phe Leu His Ser Phe Thr Met Tyr Lys Leu Tyr Gln Lys Leu

370

375

380

Phe Leu Glu Met Ile Gly Asn Gln Val Gln Ser Val Lys Ile Ser Cys

385

390

395

400

Leu

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